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ANALYSIS OF NEW PSYCHOACTIVE SUBSTANCES USING SECONDARY CALIBRATORS

Application of gas chromatography hyphenated with time-of-flight mass spectrometry and nitrogen chemiluminescence detection

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ACADEMIC DISSERTATION

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I. Ojanperä I, Mesihää S, Rasanen I, Pelander A, Ketola RA (2016) Simultaneous identification and quantification of new psychoactive substances in blood by GC-APCI-QTOFMS coupled to nitrogen chemiluminescence detection without authentic reference standards. *Anal Bioanal Chem.* **408**:3395-3400.

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ABBREVIATIONS

α-PVP	α-pyrrolidinopentiophenone
APCI	atmospheric pressure chemical ionization
CAD	charged aerosol detector
CLND	chemiluminescence nitrogen detector
CV	coefficient of variation
DIA	data-independent acquisition
EI	electron ionization
ELSD	evaporative light scattering detector
EMA	European Medicines Agency
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
ESI	electrospray ionization
FDA	US Food and Drug Administration
FID	flame ionization detector
GC	gas chromatography
GCxGC	two-dimensional gas chromatography
HRMS	high-resolution mass spectrometry
IPCS	International Programme of Chemical Safety
LC	liquid chromatography
LLE	liquid-liquid extraction
LOQ	limit of quantification
LRMS	low-resolution mass spectrometry
MBTFA	*N*-methyl-bis-trifluoroacetamide
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MSTFA	*N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide
NCD	nitrogen chemiluminescence detector
NMR	nuclear magnetic resonance
NPD	nitrogen-phosphorus detector
NPS	new psychoactive substance(s)
PMT	photomultiplier tube
PRS	primary reference standard(s)
QTOFMS	quadrupole time-of-flight mass spectrometry
RID	refractive index detector
TDM	therapeutic drug monitoring
TFAA	trifluoroacetic anhydride
TMCS	trimethylchlorosilane
TMS	trimethylsilyl group
UNODC	United Nations Office on Drugs and Crime
UHPLC	ultra-high performance liquid chromatography
UV-DAD	ultraviolet-diode array detector
VUV	vacuum ultraviolet detector

ABSTRACT

Legal medicines and illegal drugs play a significant role in today's society. The laboratory analysis of these substances is central not only in therapeutic monitoring but also in clinical and forensic toxicology, which aims to provide evidence-based information on the abuse and harmful effects of these substances. The rapid emergence of newly abused drugs known as new psychoactive substances (NPS) has challenged the conventional drug testing concept. On the European illicit drug market, approximately fifty new substances are identified each year, many of which are poorly characterized for their pharmacological effects or toxicity. Moreover, the lack of authentic primary reference standards (PRS) bottlenecks the development of analytical assays for NPS, which hinders both detection and toxicological evaluation of these drugs.

High-resolution mass spectrometry (HRMS) has recently been applied to tentative identification of unknown compounds by targeting the precursor ions with high mass accuracy, resolution and speed. This capability has permitted a breakthrough in facile screening for suspected NPS, but until now, no practical method has been described for quantitative bioanalysis of NPS in the absence of PRS. In this thesis, a new analytical platform was developed and exploited for the simultaneous qualitative and quantitative analysis of NPS and metabolites without using PRS. In this platform, the gas chromatographic (GC) flow was divided between an atmospheric pressure chemical ionization - quadrupole time-of-flight mass spectrometer (APCI-QTOFMS) for tentative identification and a nitrogen chemiluminescence detector (NCD) for quantitative estimation based on the detector's equimolar response to nitrogen-containing substances. Replacement of the ordinary electron ionization (EI) source with APCI was proven to be useful in substance identification by QTOFMS because it allowed the preservation of the precursor ion for tentative identification. The GC-APCI-QTOFMS tandem mass spectrometry (MS/MS) experiments using 29 NPS showed that all substances shared the same major fragments as in the commercial spectral library created by using liquid chromatography (LC) electrospray (ESI) MS/MS instrumentation. Consequently, these findings promote the usability of external soft-ionization compound libraries with the new GC-APCI-QTOFMS platform. The accuracy and precision of the *N*-equimolar quantification by GC-NCD were tested using several NPS in four separate studies using post-spiked sheep blood (n = 5), post-mortem blood (n = 38) and urine (n = 3), and in seized powdery material (n = 28). The combined results from these studies, excluding the post-spiked samples, showed that NPS could be quantified with a grand mean accuracy of 91.7% and with a grand mean imprecision (CV) of 9.5% in the absence of PRS for compensating sample preparation and analysis.

In conclusion, the GC-NCD-APCI-QTOFMS platform proved feasible for analyzing suspected nitrogen-containing drugs, such as stimulant-type NPS and metabolites, in situations where appropriate reference standards are not readily accessible. Simultaneous tentative identification by HRMS and quantitative estimation by NCD has unparalleled potential for fast preliminary analysis until a certified PRS becomes available. The

possibility for high-throughput retrospective identification and quantification is an additional advantage of this platform, providing a means for further research on emerging NPS.

1 INTRODUCTION

Drug testing is one of the most commonly employed branches of analytical and forensic toxicology, used to provide clinical, supervisory or judicial evidence for potential substance abuse or poisoning. Analysis of drugs of abuse may help to answer questions such as: What was the cause of death? Was the person under the influence of alcohol or drugs? Was the victim drugged? What is the quantity and purity of seized illegal substances? On a population-scale, further analysis of forensic drug testing data can be used to study epidemiological patterns, such as the prevalence of drug use in the society, and link certain drugs with a higher incidence of toxicity. According to the basic principles of pharmacology and toxicology, the induced effect mediates through two interconnected factors: mechanism of action and quantity. Thereby the knowledge of the presence of a drug in the body is not alone sufficient to understand the magnitude of its effect in a given situation, although it may be sufficient for administrative sanctions. In forensic toxicology, quantitative analysis is employed to distinguish impairment from non-impairment in many types of investigations, such as driving under the influence or cause-of-death investigation.

Over the past few decades, one of the most challenging problems in forensic toxicology has been the accelerated emergence of new psychoactive substances (NPS), which were formerly known as designer drugs. The United Nations Office on Drugs and Crime (UNODC) has defined NPS as “substances of abuse, either in a pure form or a preparation, that are not controlled by the 1961 Single Convention on Narcotic Drugs [1] or the 1971 Convention on Psychotropic Substances [2], but which may pose a public health threat” [3]. According to the EU Drug Markets Report 2019, the EU Early Warning System reported 172 new substances between years 2016-2018, with an approximal annual rate of 57 NPS per year [4]. Attempts to legislatively control NPS have been slow since drug policy in most countries has been adapted from these UN treaties, which were not designed for facing numerous NPS. The legislative problems related to NPS are often characterized as a cat-and-mouse game, meaning that new substances appear as soon as established NPS are banned. Additionally, these substances’ hazardous effects are not well understood because comprehensive drug safety testing has not been carried out. Moreover, keeping up with the emerging trends is difficult because of fast production and a short lifetime of NPS.

The analytical measurement of NPS is difficult, because certified primary reference standards (PRS) are often unavailable. In conventional drug analysis, a reference standard is needed to prove drug identity based on the unique chemical properties of a substance under defined experimental conditions, especially the retention time in chromatographic analysis and the fragmentation pattern in mass spectrometry (MS). In quantitative analysis, reference standards are used to construct a calibration curve. Rapid detection of NPS has been drastically facilitated by the advent of high-resolution mass spectrometry (HRMS) as a part of modern wide-scope screening methods, enabling simultaneous detection of various substances at a ng/mL level in complex biological samples, such as blood or urine [5, 6]. However, quantification using this instrumentation is problematic because the production of ions in the ion source is structure-dependent, and the signal response is highly unpredictable without proper reference standards [7, 8]. For this reason, clinical and forensic laboratories, as well as the scientific literature, lack quantitative reference data for most NPS and especially for their metabolites. A rare but exciting approach is to apply the nitrogen chemiluminescence detector (NCD) to the quantitative estimation of nitrogen-containing drugs in the absence of PRS [9]. This detector possesses an equimolar response to nitrogen, and therefore, a suitable external nitrogen-containing calibrator can be used for quantitative calibration. Until now, drug analysis by NCD has been limited to liquid chromatography (LC) applications, however, gas chromatography (GC) could potentially be a more useful technique in the analysis of complex biological material due to its better chromatographic resolution.

In this thesis, a new concept of combining HRMS with NCD for the analysis of NPS and metabolites without PRS is presented and elaborated. In the analytical platform studied, the GC flow is divided between an NCD for quantification and a quadrupole time-of-flight mass spectrometer (QTOFMS), equipped with an atmospheric pressure chemical ionization (APCI) source, for identification. This approach is anticipated to be feasible in NPS analysis, because most drugs and psychoactive substances contain nitrogen in their structure, and consequently the approach allows instant filtering of non-nitrogen compounds in complex samples. The studies included in this thesis explore the possibility of using the GC-NCD-APCI-QTOFMS platform as an integral part of customary forensic applications, such as drug screening in biological specimens or purity analysis of seized drugs. In this context, the laboratory would be able to obtain quantitative information on new substances as soon as they become available on the illegal market via the Internet.

2 REVIEW OF THE LITERATURE

2.1 DRUG TOXICITY

Consumption of psychoactive substances has been historically documented in the context of medical use (e.g., opium), in religious ceremonies (e.g., *Amanita muscaria*), and in socially acceptable products (e.g., alcohol and tobacco) [10]. Since the 20th century, the pharmaceutical industry has given rise to numerous new synthetic drugs that are available to today's healthcare. At the same time, the prevalence of the abuse of prescription drugs and illicit drugs is increasing. In Finland for instance, drug-related mortality has almost doubled between 2000 and 2018 (from 2.6 to 4.7 deaths per 100 000 capita) [11].

In some cases, even medicinal drug intake causes unintended adverse effects, and therefore healthcare professionals must choose appropriate medical treatment by balancing the benefits over the risks. According to the definition by the International Programme of Chemical Safety (IPCS), the risk can be defined as "the probability of an adverse effect in an organism, system, or (sub)population caused under specified circumstances by exposure to an agent." [12]. Adverse effects and exposure can be studied by the examination of pharmacodynamic (what drug does to body) and pharmacokinetic (what body does to drugs) properties of a drug. The study of pharmacodynamics reveals the mechanisms of the therapeutic and adverse effects of a drug that take place by modulating the body's endogenous machinery (i.e. potency, receptor selectivity, binding affinity, or type of agonism). In contrast, pharmacokinetic factors control the exposure to a drug at the site of action by various mechanisms (i.e. absorption, distribution, metabolism, excretion or protein binding) [13]. Consequently, the complexity of possible molecular interactions explains why drugs with similar molecular structures exhibit varying physicochemical properties that result in varying responses, such as the onset and duration of action, potency, adverse effects or even pharmacological response. Naloxone and morphine, for example, are structurally similar yet their action in the opioid receptors is reverse.

There is a growing interest in conducting toxicological studies faster and more cost-effectively by using a combination of *in vitro* tests, high-throughput analytical techniques and computational *in silico* methods [14]. In practice, examination of the drug structure-function relationship allows rapid pharmacological classification of drugs, and modern *in silico* tools can use structural data to predict potential functional or toxicological signals at the early phase of drug development or toxicological assessment [15, 16]. Despite the undisputed benefits of structural prediction, the lengthy and resource-intensive safety testing of new drug candidates is still likely to remain in a central position in the assessment of potential toxicity.

2.1.1 ROLE OF QUANTITY IN TOXICITY AND FORENSIC ANALYSIS

In his defense against accusations of treating patients with toxic mercury, Paracelsus, a famous Swiss physician and alchemist replied with a sentence that would later become a famous quote in toxicology: “What is there that is not poison, all things are poison and nothing (is) without poison. Solely the dose determines that a thing is not a poison” [17]. According to Paracelsus, treatment is only successful if the dose is neither too much nor too little. This idea has been well adapted in modern pharmacotherapy, where the dose-response relationship is carefully investigated to reduce the incidence of adverse effects. One widely used method to characterize a suitable dose has been the use of the therapeutic index, in which a range of doses is tested to observe both the minimum therapeutically effective dose and the dose that produces adverse effects [18]. A similar concept is used in the clinical practice known as the therapeutic drug monitoring (TDM), where physicians monitor the drug concentration levels of their patients at specific intervals to ensure that effective dose is given continuously with minimal adverse effects [19, 20].

It is evident that drug concentrations should be measured from forensic samples (e.g., blood, urine or seized material) always when it is crucial to determine the magnitude of drug effect, impairment, or in the valuation of illicit drug seizures. In the light of the fact that quantitative assays are always more tedious, costly and time-consuming than identification alone, such analyses must provide additional value to forensic decision-making, some of which are exemplified in table 1.

Table 1. Common incentives for drug quantification in the scope of toxicology and forensic medicine.

Application	Example of analytical target(s)	Use or impact on decision	Reference
Pharmaceutical and biomedical			
Pharmaceutical purity analysis	Pharmaceutical product	Quality control	[21]
Drug safety testing	New pharmaceutical product in blood	Estimation of safe and effective dose	[22, 23]
Therapeutic drug monitoring (TDM)	Lithium or cyclosporine in blood	Determination of appropriate dose	[19, 20]
Forensic science			
Police investigation	Seized illicit drugs	Information on drug trafficking trends	[24]
	Seized illicit drugs	Classification of drug offence	[25]
Monitoring of abstinence treatment	Ethyl glucuronide in urine	Indication of recent ethanol intake	[26]
	Benzodiazepines in hair	Indication of intake at certain time interval	[27]
Traffic satefy	Ethanol and drugs in blood	Indication of impairment	[28]
Drug abuse confirmation	Cannabis or γ-hydroxybutyrate in urine	Positive/Negative result based on cut-off values	[29, 30]
Post-mortem toxicology	Drugs in post-mortem blood	Evidence in determination of cause of death	[31]
	Opioids and their metabolites in blood	Indication of acute overdose	[32]

2.1.2 DRUG METABOLITES

Foreign molecules (xenobiotics) are eliminated mainly through urine and feces [33]. During the clearance many drugs are transformed into water-soluble metabolites, mostly in the liver, kidney and intestines [34]. The enzymatic reactions catalyzing drug transformation are divided into phase I (functionalization) and phase II (conjugation) reactions. In phase I, one or several separate reactions (e.g., oxidation, reduction, or hydrolysis) are required to reveal a functional group that is compatible to form a covalent bond with a polar endogenous conjugating agent (e.g., gluthathione or uridine diphosphate glucuronic acid) in phase II [34]. It is estimated that approximately three-quarters of the top 200 prescribed drugs in the US (in the year 2002) undergo a metabolic transformation in the human body, often by the catalytic action of the cytochrome P450 enzyme family [35]. As a result, unchanged drugs, drug metabolites, or a mixture of both are detectable in body fluids.

Failure in drug metabolism can lead to unexpected toxicity due to accumulation or temporary increase or decrease of the drug concentration at its target location. It is now well established that drug metabolism is sensitive to many external and internal factors (e.g., genetic variation, age-specific expression of metabolic enzymes, cellular damage or modulation of metabolizing enzymes), which may expose drug therapy to risk [34, 36]. Particularly drug-drug interactions in polydrug use are commonly causing adverse effects or even fatal poisonings in geriatric patients and recreational drug users [13, 37, 38]. In some cases, metabolites themselves can induce acute or chronic toxicity (e.g., via depletion of the detoxification mechanisms) [39]. Furthermore, it is estimated that a reactive metabolite, rather than the parent drug itself, is responsible for most of the idiosyncratic adverse effects that are not necessarily detected in large clinical trials [40, 41]. In the 2000s, the safety testing of drug metabolites has gained increasingly more attention in pharmaceutical development. Much of the groundwork was laid in a paper by Baillie *et al.* (2002), suggesting that drug safety testing should include major human metabolites [42]. In this particular proposition, quantitative metabolite safety testing was suggested to be carried out if the metabolite is considered a "major metabolite" (>25% of drug's total exposure), if it is an important active metabolite which is known to contribute to the pharmacological activity of its parent drug, or if a metabolite contains a structural alert for toxicity. Consequently, some of these suggestions were adapted in the official guidelines, such as by the European Medicines Agency (EMA 2013) and the US Food and Drug Administration (FDA 2020), where it is stated that major drug metabolites that contribute greater than 10% of drug's exposure should be tested [43, 44].

Metabolites are richly encountered in urine. Urine is easy to collect, and drugs in urine have generally a longer detection time-window compared to blood [45]. These features make urine an ideal specimen for qualitative forensic analysis, such as wide-scope drug screening. However, quantitative data obtained from urine analysis must be interpreted with caution, because absolute concentration values are largely influenced by changes in water content, pH, metabolic processes and time of drug intake [46]. Recognizing these variables, the establishment of method-specific, administrative cut-off concentration values is often employed in urine drug testing to aid the interpretation of results and decision-making [47].

Quantification of drug metabolites can provide conclusive information in forensic casework. For instance, the metabolite to parent drug concentration ratio has provided valuable information in the interpretation of forensic or clinical toxicology cases, including the estimation of timing of single-dose drug intake [48], investigation of adherence to treatment [49], determination of recent alcohol consumption [50], distinguishing between heroin and codeine use among individuals arrested for driving under the influence of drugs cases [51] or investigation of the manner of death due to acute drug toxicity [32, 52]. Beyond forensic sciences, quantitative metabolomic profiling is regarded as a promising approach to identify phenotypic changes in an organism, which could be used to study molecular mechanisms of various diseases and enable the development of prognostic and diagnostic tests based on specific patterns in metabolite levels [53, 54].

2.2 NEW PSYCHOACTIVE SUBSTANCES (NPS)

The NPS consist of diverse groups of substances that have been synthesized to mimic the anxiolytic, euphoric or addictive properties of illicit drugs. In a broad pharmacological sense, NPS can be categorized as depressants (including benzodiazepines, opioids and dissociatives), hallucinogens, stimulants, synthetic cannabinoids and “others” that do not clearly belong to any of the former groups. Some Internet vendors are typically openly marketing NPS with euphemisms, such as “legal highs” or “research chemicals”, referring to the fact that the new substances are intended to circumvent existing drug legislation. For practical reasons, the scientific literature is retaining the term NPS also for those NPS that are already under legislative control in some countries. Besides the legal or perceived legal status, the consumption of NPS is driven by other factors such as their ready availability, non-detectability in conventional drug tests, inexpensive dose price, perception of higher purity, and exciting user experience [55, 56]. Some NPS persist in the drug market even after legislative control [57], but many of these substances swiftly enter and exit the drug market, which leaves a minimal time-window for public health interventions. Healthcare and legislative response to NPS is challenging due to the high rate of emerging substances and their availability through the Internet. According to the UNODC, one or several NPS have been encountered already in 120 countries and territories with a total number of over 950 substances by December 2019 [3]. On the EU drug market, the number of monitored NPS has already risen to over 730 substances [4]. Furthermore, the rate of newly reported substances sharply increased in the 2010s when 41 to 101 new substances were annually reported [4]. In recent years, a worrying phenomenon has emerged involving mislabeled NPS [58, 59]. NPS have also been used as cutting agents for traditional illicit drugs [60]. Such an activity has been frequently observed with stimulant-type NPS that have been sold as amphetamine or MDMA [61, 62], but also in cases where highly potent fentanyl analogs have been sold as heroin [63]. Especially in the latter case, the risk of fatal overdose is apparent due to misidentification and the difficulty of measuring an appropriate dose, both of which can lead to fatal respiratory depression [64-66]. These criminal actions might be explained on grounds of a better cost-efficiency or a lower risk of the product being detected in drug screening.

The scarcity of information on the fundamental pharmacology and recreational and toxic concentration levels of NPS, combined with the obscurity of drug action due to polydrug use, makes evidence-based counter-measures difficult [67, 68]. Currently, only a small percentage of NPS have been studied under preclinical conditions, and even fewer have been tested on humans [69]. Furthermore, most of the available preclinical studies have been narrowly focused on the addictive potential, while a distinctively smaller number of studies are linked to chronic or acutely hazardous effects, such as cardiotoxicity, respiratory depression, neurological symptoms or hyperthermia [68]. In order to mitigate these harms, a responsive international collaboration system has been established. The EU Early Warning System, operated by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) and Europol, plays a central role in supporting national and EU-level preparedness and responses to NPS. In operation since 1997, it comprises those two agencies, 30 national early warning systems across Europe, the European Medicines Agency and the European Commission. The Early Warning System is the first step in a three-step legal framework, additionally comprising risk assessment and legislative control [70, 71]. The purpose of the EU Early Warning System is to enable rapid information exchange on detection, prevalence and harmfulness of emerging NPS between the EU member states, as well as Norway, Turkey and United Kingdom.

Forensic laboratory analysis plays a central role in getting off to a good start in understanding the epidemiology and toxicology of NPS. However, a recent report by the EMCDDA points out that laboratories continue to struggle with the analytical testing of NPS because of the unavailability of certified or otherwise reliable reference standards [72]. Therefore it has become evident that the transient nature of NPS and their fluctuation on the illicit drug market are hampering both legislative and scientific responses that are related to emerging NPS [73]. In general, access to these standards is hindered by multiple time-consuming steps that should be completed before an analytically valid laboratory method can be put into operation (Figure 1). Initially, a sample of the suspected NPS should be acquired, followed by structural elucidation using advanced laboratory techniques, such as nuclear magnetic resonance spectroscopy (NMR) and HRMS [74]. Next, the structural information on the NPS released by the EMCDDA Early Warning System network can be used for early tentative detection by HRMS, until PRS are commercially available.

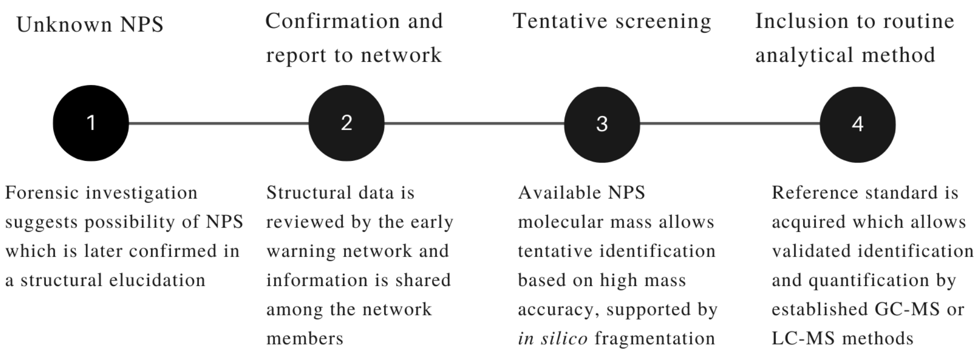


Figure 1. Time-course for the development of analytical testing of NPS.

2.2.1 STIMULANTS

Stimulants are globally the largest group of NPS, as they represent approximately 36% of all NPS (December 2019) [3]. Since the rapid increase of reported NPS in the mid-2000s, some of the stimulant-type NPS have gained popularity as legal alternatives to the more established stimulants, such as amphetamine, methamphetamine, MDMA, or cocaine. Following this period, synthetic cathinones, sometimes marketed as “bath salts”, have become the most common stimulant-type NPS group, representing 39% of the overall NPS seizures in the EU in 2017 [4]. Synthetic cathinones are structural analogs of cathinone, a substance found in the khat plant (*Catha edulis*). As with other stimulants, they are commonly sold as powders and consumed orally or by snorting, but also injected intravenously [4]. Evidently, the emergence of various stimulant-type NPS has brought new problems, such as adulteration of the established illegal stimulants with synthetic cathinones [61] and fast-paced changes in the street drug composition.

The main pharmacological mechanism of action of stimulant-type NPS is executed through the release of dopamine, noradrenaline and serotonin in varying levels. Typically desired stimulant effects are elevated alertness, wakefulness, concentration, and altered behavioral characteristics, such as euphoria, self-confidence and aggressiveness. Adverse effects include drug dependence, violent behavior, tachycardia, arrhythmia, kidney or liver failure, skin rashes, hypertension, hyperthermia, hallucinations, seizures and stroke [75, 76]. Structural classification of stimulant-type NPS is difficult because the members of this pharmacological class exhibit great structural diversity that may or may not have a resemblance to the more established drugs (Figure 2). Moreover, the aforementioned pharmacological properties of stimulant-type NPS overlap with other commonly encountered NPS classes, such as synthetic cannabinoids and hallucinogens.

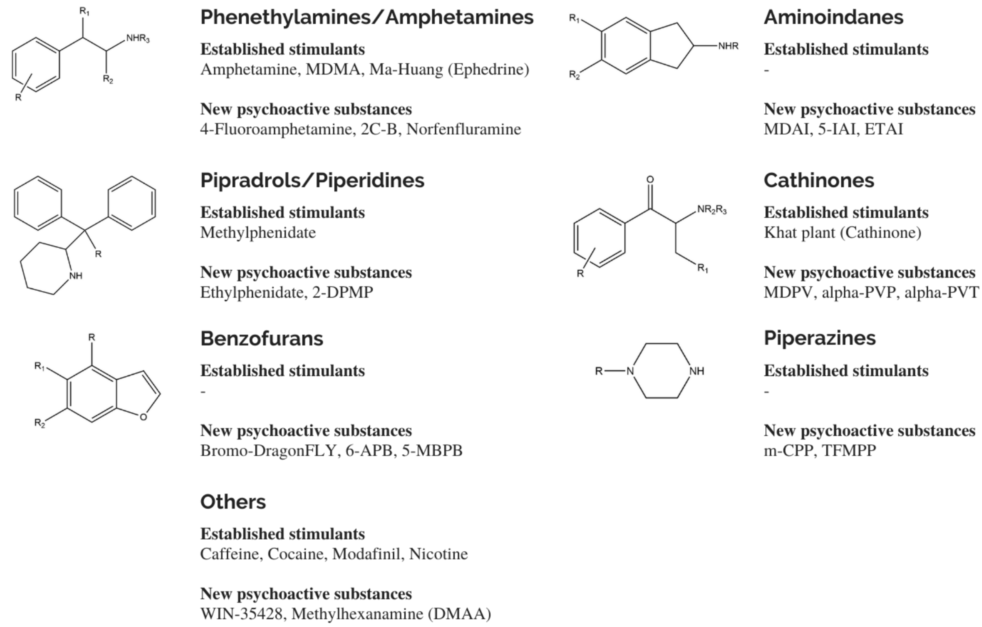


Figure 2. Structural classification of NPS stimulants and related established stimulants. Adapted from Miliano et al. 2016 [77].

2.3 ANALYSIS OF NEW PSYCHOACTIVE SUBSTANCES WITHOUT PRIMARY REFERENCE STANDARDS (PRS)

The term PRS refers to an authenticated, uniform material that is intended for use in specified chemical analysis, in which its properties are compared with those of the sample under examination. A PRS possesses a degree of purity usually of 99.5% or higher.

Traditionally, validity is determined experimentally by measuring accuracy (proximity to the true value) and precision (consistency of repeated measurements). Traceability and uncertainty are more recently introduced concepts, increasingly emphasized in the quality assurance guidelines and certification requirements (e.g., ISO/IEC 17025) [78]. The metrological traceability is defined as the "property of a measurement result whereby the result can be related to a reference through a documented unbroken chain of calibrations, each contributing to the measurement uncertainty" (International Vocabulary of Metrology, 2007) [79]. In essence, it describes the comparability of individual measurements in different laboratories or at other time points, which is determined by the degree of uncertainty at each step.

Lack of PRS is an evident problem in the analysis of NPS and their metabolites, compromising the traceability of results. The rapid emergence of a large number of NPS and their short lifetime oblige forensic laboratories to acquire numerous new reference standards, preferably within the shortest time period possible. However, PRS are often expensive, and some of them do not even exist in the catalogs of commercial vendors. Some vendors offer custom synthesis services, however, these are even more costly and the acquisition of such material may take even 3 to 5 years [80]. Alternatively, NPS reference standards can be sought by gray-market sourcing (direct purchase from an NPS vendor) or by utilizing pharmaceutical impurities [81]. However, these type of material are not directly suitable for quantitative analysis, because the product content is not reliable and the purity can be either low or inconsistent.

Presently, a combination of HRMS and NMR techniques is a favored strategy in the structural elucidation of unknown NPS, as these techniques can uncover highly specific chemical information about the substance in question (e.g., molecular composition, molecular structure, molecular mass, functional groups, connectivity of atoms) [74]. In addition, NMR can be used to estimate the purity of unknown drug material [74, 82]. Despite the merits of NMR in structure analysis, this thesis focuses on HRMS which is a more achievable technique with a capability for high-throughput analysis of complex biological specimens.

2.3.1 HIGH-RESOLUTION MASS SPECTROMETRY (HRMS): IDENTIFICATION OF UNKNOWN SUBSTANCES

Chromatographic and MS techniques have taken root in today's clinical and forensic laboratories that analyze body fluids, tissues and seized material. Because of their sensitivity, selectivity and high-throughput capability, hyphenated MS techniques have become indispensable in drug analysis [83], increasingly surpassing immunoassay techniques [84, 85]. Both GC-MS and LC-MS are capable of producing a vast amount of information in a single analysis, which makes these techniques ideal for the fast and comprehensive screening of hundreds of drugs, metabolites and biomarkers. In MS, identification can be pursued with or without using a PRS. When reference standards are available, the experimentally determined properties of an analyte can be compared with the corresponding properties of a reference standard. Identification parameters, such as the retention time, isotopic pattern, monoisotopic mass of precursor ion, and spectral pattern can be stored in an in-house spectral library or database. In the absence of reference standards, readily available MS/MS libraries or databases can be used to identify target substances that are experimentally fragmented under similar analytical conditions. However, identification is less accurate, if external spectral libraries or databases are used under poorly matching conditions without retention time information. On the other hand, a mere knowledge of the molecular structure may be sufficient, when identification is based on the accurate mass measurement of the precursor ion, comparison to theoretical *in silico* mass spectra, or interpretation of molecular fragmentation by using rules and algorithms [86].

General unknown screening is considered one of the most challenging tasks in forensic laboratories, because the analyst has to develop generic analysis methods and mine complex MS/MS data sets. However, the initial detection of NPS is efficiently carried out using an analytical strategy known as "suspect screening", in which the tentative identification is based on preliminary information on molecular formula to suggest substance identity [87-89]. The MS-based suspect screening approach has made progress over the last few decades, firstly because of the advances in HRMS, and secondly due to the emergence of a new data collection technique called data-independent acquisition (DIA). The advent of new HRMS analyzers has enabled fast, straightforward and affordable tentative substance identification, based on accurate (± 5 ppm) molecular mass measurement. Suspect screening is based on the fact that if the mass of an ion from a chemical compound is determined with sufficient accuracy, the elemental composition of that compound can be deduced. Low-resolution mass spectrometry (LRMS), on the other hand, is restricted to aid identification mainly based on fragmentation patterns. Unlike Fourier transform ion cyclotron resonance MS (FT-ICR), which is still recognized as the state-of-the-art technique in terms of mass accuracy and resolving power, current quadrupole time-of-flight MS (QTOFMS) and Orbitrap MS techniques are more suitable for routine analytical laboratories because of lower costs and more facile operation. High data acquisition speed is required in the hyphenated techniques to ensure the possibility of acquiring multiple data points across the chromatographic peak. The improved data acquisition in DIA enables the collection of precursor and fragment data by alternating the collision energies throughout the chromatographic separation, without predefining target molecules in the acquisition method. DIA was exploited already in 2001 in the context of target and suspect drug screening with use of a single stage LC-TOFMS instrument by Gergov et al. (2001) [90], and more recently, DIA based LC-QTOFMS has been increasingly applied to the early detection of drugs, NPS and metabolites in herbal blends [91] and biological samples [6, 92].

Evidently, HRMS together with DIA allow a better sensitivity and previously unavailable advantages in drug analysis, such as the tentative identification based on molecular formulae (elemental composition) and retrospective analysis. These features are certainly ideal in the suspect screening for NPS. In retrospective analysis, historical acquisition data is re-processed once new suspect information is obtained. For example, Noble et al. (2018) stored the HRMS data from 2339 blood samples and re-processed the original data against an updated database containing 50 fentanyl analogs [93].

Identification of unknown substances by HRMS is facilitated by the availability of several online spectral libraries, as the acquired data can be searched against the spectral information that is provided by other investigators. Some examples of the existing free libraries or databases are MassBank [94], mzCloud [95], NPS Data Hub [96], and the recently emerged HighResNPS [97]. Interestingly, HighResNPS utilizes a database containing the diagnostic fragment ion instead of the whole spectrum. This feature promotes better inter-laboratory transferability, as the search results are less affected by different method variables and HRMS vendor platforms. A vast majority of the accurate mass –based spectral data commonly available has been collected using LC combined with ESI and HRMS, while comparable data by GC-APCI is lacking.

2.3.2 SEMI-QUANTITATIVE MASS SPECTROMETRY

Thus far, there have been no studies attempting to measure NPS in biological samples accurately in the absence of PRS or radiolabels. In semi-quantitative analysis, an approximation of the concentration is obtained by assuming that the ion response of a target substance is similar to a secondary calibrator. Overall, semi-quantitative analysis by MS can provide a quick approximation of analyte concentration that could be incredibly valuable in an early phase of research. This approach remains inaccurate because the ionization efficiency is sensitive to the analytical conditions, surrounding matrix, and analyte structure [7, 8, 98]. Furthermore, the extraction recovery cannot be determined in the absence of reference standards. However, according to Niwa et al. (2020), the role of extraction recovery is relatively insignificant when using a structurally similar secondary calibrator. In their report, approximately 86% of the measurement bias was attributed to the ionization response ratio, and only 14% was caused by a loss in the sample extraction stage [98].

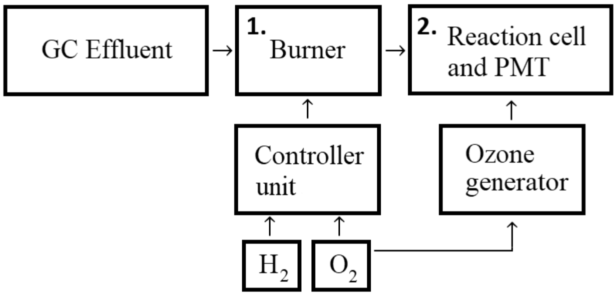
Despite the fact that the use of secondary calibrators attempt to minimize measurement bias, previous studies with LC-ESI-HRMS have shown that a gross error in quantitative estimation should be expected, if an equal signal response is assumed. In the study by Hatsis et al. (2017), the parent drugs were used as surrogates for the quantitative estimation of their metabolites. In their dataset of 45 parent drugs and metabolites prepared in a solvent, more than a half of the studied substances were quantified with over 2-fold error, the error being up to 71-fold in the worst case [8]. A bias of the same magnitude was found with Orbitrap and QTOFMS instrumentation in the study by Blanz et al. (2017), in which the measurement bias for 199 out of 233 (85%) measured metabolites ranged 20-200%, while the rest of the metabolites were either outside this range or remained undetected [99]. Kruve et al. (2020) improved the quantitative estimation approximately by 10-fold using an instrument-corrected ionization prediction model, in which predictions were made based on analyte structure and solvent composition. Using this *in silico* model, a 5.4-fold mean measurement bias was obtained for a set of 31 substances that were spiked into blank oat, barley, rye, wheat, rice and maize [100]. The use of LC-nanospray ionization MS has been a promising improvement towards uniform response, because it exhibits a higher tolerance to salts and shows a better ionization efficiency than the conventional ESI, reducing the overall bias of the ionization process [101]. Nanospray was used by Valaskovic et al. (2006) for the quantitative estimation of the well-known drug metabolites of codeine, dextromethorphan, tolbutamide, phenobarbital, cocaine and morphine. All metabolites were measured accurately without PRS (20% bias), when parent drugs were used as secondary calibrators [102]. However, less satisfactory data were obtained in the comprehensive study by Schadt et al. (2011), in which 73 metabolites in various matrices were quantified using the corresponding parent drug as a secondary calibrator [103]. In this study, 86% of the metabolites could be measured within a 300% bias range. Clearly, these semi-quantitative estimations fall short of the guidelines used in forensic toxicology, where usually only a 20% bias is tolerated in the basic method validation [104].

2.3.3 NITROGEN CHEMILUMINESCENCE DETECTION (NCD)

The NCD is one of the most viable detectors for the quantitative analysis of drugs without PRS, because it possesses an equimolar response to nitrogen [105]. When combined with LC, the technique has been referred to as chemiluminescence nitrogen detection (LC-CLND). Although other universal (or near-universal) detection approaches exist, including the LC-coupled refractive index detector (LC-RID), evaporative light scattering detector

(LC-ELSD), and charged aerosol detector (LC-CAD), as well as the GC-coupled vacuum ultraviolet detector (GC-VUV), flame ionization detector (GC-FID), these techniques have shown very restricted use in the analysis of biological samples due to their limited sensitivity or limited capacity to analyze complex material, as reviewed by Zhang et al. (2019) [106]. Quantitative NMR stands out because it allows accurate and precise quantitative estimation without requiring an isolation procedure [107]. The problems encountered in the past, including poor linearity, stability and sensitivity, are significantly improved with modern NMR instruments [106]. However, occasional data loss caused by overlapping resonances and the complexity of data analysis may still limit its broader application to the analysis of biological matrices [107, 108].

Many known small molecules, such as vitamins, nucleic acids, natural alkaloids and drugs, contain nitrogen atoms in their structure and exhibit biological activity or pharmacologically interesting properties [109]. According to a database of MDL Information Systems, approximately 90% of pharmaceutical drugs contain nitrogen [110], making NCD an attractive option for drug analysis. Major drugs of abuse that do not contain nitrogen are few; they include gamma-hydroxybutyrate, natural and many synthetic cannabinoids, as well as most anabolic steroids. Quantification by NCD is based on the chemiluminescence reaction, in which the measured signal is an emission that is released after nitrogen compounds are oxidized to nitric oxide and subsequently reacted with ozone in the gas phase (detailed operating principle in Figure 3) [111]. The emitted chemiluminescence has a high linear range and is equimolar to nitrogen [105]. According to the manufacturer's specifications, a commercial GC-NCD instrument has a favorable linearity ($>10^4$), sensitivity (LOD < 3 pg nitrogen/s) and selectivity over carbon signal (> $2x10^{7}$ response N / response C) [112]. This mechanism essentially differs from the nitrogen-phosphorus detector (NPD), in which the signal correlates with the number of produced ions [113]. More specifically, NCD is not a universal detector, because minor structure-dependent response have been observed. However, only a 10-15% variation has been reported in all cases, except when two nitrogen atoms are adjacent to each other [114].



1. Combustion of nitrogen compounds

$$R\text{-}N + O_2 \xrightarrow[900\ ^\circ C]{} NO + CO_2 + H_2O$$

2. Ozone-induced chemiluminescent detection of nitrogen

$$NO + O_3 \xrightarrow[\text{excitation}]{} NO_2^{*} + O_2$$

$$NO_2^{*} \xrightarrow[\text{relaxation}]{} NO_2^{\circ} + h\nu \ (>800\ nm)$$

Figure 3. Schematic diagram of gas chromatography - nitrogen chemiluminescence detection (GC-NCD). The GC effluent is directed to the burner (1), where in the presence of oxygen at a high temperature (900 °C), organic molecules are combusted to various oxides and water. Nitric oxide is transferred to the reaction cell (2), where it is mixed with ozone (O_3) in order to produce nitrogen dioxide in its excited state (NO_2^{*}). Next, nitrogen dioxide returns to the ground state with the emission of photons. The resulting photocurrent is amplified in the photomultiplier tube (PMT) and detected. The Figure was adapted from Agilent 255 NCD Operation and Maintenance Manual (2012) [112].

Prior to 1980's, commercial standalone NCD instruments were mainly applied to environmental analysis [115, 116], but a leap towards drug and bioanalysis was taken upon the appearance of hyphenated GC-NCD in 1980 [117], and later with LC in 1988 [118]. In their analysis of dried tobacco samples, Cai et al. (2012) [119] pointed out that GC-NCD exhibited a superior precision, stability and peak shape over GC-NPD. The investigation of eight tobacco alkaloids in five replicate injections (0.5 µg/mL) showed that the CV was generally lower by GC-NCD (<0.5%) than by GC-NPD (<3.2%), possibly because of the NPD's susceptibility to solvent interferences and bead aging. It was also mentioned that one alkaloid could be used to quantify the others by using the *N*-equimolar principle, which could significantly simplify the experimental set-up. Based on LC-CLND, the *N*-equimolar response has been used in a range of applications to determine the purity and quantity of small molecule drugs and organic compounds. Fitch et al. (1997) proposed the use of LC-CLND for the determination of the yield and purity of synthesis products [120], and this idea was later applied by Yan et al. (2003) [121] and Letot et al. (2005) [122] to combinatorial chemistry libraries using caffeine and diphenhydramine, respectively, as external secondary calibrators. Thomas et al. (2016) applied LC-CLND to estimate the purity of several toxin reference standards using caffeine as an external secondary calibrator. In that study, the purity of seven structurally diverse natural toxins was measured with an impressive accuracy of 97-102% and a high precision (CV 2%) [123].

Apart from the articles **I-V** included in this thesis, *N*-equimolar quantification of NPS by NCD/CLND has been published only in three other papers, all of which focused on the analysis of drugs seized by the Finnish police or Customs, using an LC-CLND instrument and caffeine as an external calibrator. In the first paper, published by Laks et al. (2004) [9], 21 seized samples, containing mostly commonly abused drugs (e.g., amphetamine, cocaine and heroin) and some tryptamine-derived NPS, were analyzed. In total, 11 individual substances were quantified by LC-CLND and the results were compared to a reference method. The mean difference compared to the reference method was 11% (range 4-21%) and imprecision (CV) was 6%. Ten years later, the LC-CLND approach was applied by Rasanen et al. (2014) to 61 previously identified NPS in 177 seized powders or herbal products, containing mostly stimulants/hallucinogens and synthetic cannabinoids. In that study, the method performance was tested using 16 NPS with known purity, resulting in 95% mean accuracy (range 83-105%) and 13% mean expanded uncertainty of measurement [124]. In the third paper, Rasanen et al. (2019) applied LC-CLND to the purity analysis of seized samples containing furanylfentanyl (N = 112), carfentanil (N = 98) and U-47700 (N = 7). In that study, the method performance was tested using 11 synthetic opioids, resulting in 96% mean accuracy (range 91-101%) [125].

Apart from the articles **I** and **III-V** included in this thesis, only three papers can be found in the scientific literature that apply quantitative LC-CLND analysis without PRS to body fluids. The study published by Deng et al. (2004), using dog urine and plasma, and the study by Ojanperä et al. (2007), using human plasma, were proof of concept -type studies determining the metabolite to parent drug concentration ratios with a simulated assumption that PRS for the metabolites were not available [126, 127]. In the third paper by Ojanperä et al. (2007), 33 drugs spiked in plasma and blood were quantified after liquid-liquid extraction (LLE) with *n*-butyl chloride - isopropanol (98 + 2) at a basic pH [128]. The mean bias in plasma and blood was 24% and 17%, respectively, and the highest bias in either matrix was 31%.

It is noteworthy that, prior to the present thesis, GC-NCD was neither applied to analytical problems related to NPS nor the combination of HRMS and NCD for simultaneous identification and quantification was explored. In particular, this thesis presents pioneering studies on the use of NCD hyphenated with GC, rather than LC, in *N*-equimolar drug analysis. Obviously, the GC-NCD-APCI-QTOFMS platform will expand the scope of the previously presented approach by enabling identification and quantitative monitoring of the desired nitrogen compounds present in either biological samples or in seized material.

3 AIMS OF THE STUDY

The aim of the thesis was to develop and validate a new analytical approach for instant simultaneous identification and quantification of suspected NPS in biological samples and in seized material, by using secondary quantitative calibrators to simulate conditions where PRS are not accessible.

The specific aims of each article were as follows:

- To describe the new GC-NCD-APCI-QTOFMS approach and verify its performance in drug analysis under preliminary experimental conditions using sheep blood (**I**).

- To elaborate accurate mass -based monitoring of suspect NPS and the usability of external drug databases by employing the GC-APCI interface (**II**).

- To develop and evaluate a quantitative analytical method for the investigation of the urinary metabolism of the stimulant-NPS alpha-pyrrolidinopentiophenone (α-PVP) using the GC-NCD-APCI-QTOFMS approach (**III**).

- To develop and evaluate a generic quantitative analytical method for NPS-stimulants in blood samples using the GC-NCD-APCI-QTOFMS approach (**IV**).

- To develop and evaluate a generic quantitative analytical method for NPS-stimulants in seized powdery material using the GC-NCD-APCI-QTOFMS approach (**V**).

4 MATERIALS AND METHODS

This chapter briefly describes the procedures of this work. More detailed descriptions are found in the original articles (**I-V**).

4.1 MATERIALS

4.1.1 REFERENCE STANDARDS AND SEIZED DRUGS

All PRS were obtained from various pharmaceutical companies and were of pharmaceutical purity. The seized material used in **II** and **V** were obtained from the Finnish law enforcement authorities (National Bureau of Investigation and Customs Laboratory).

4.1.2 SAMPLE MATERIAL

All post-mortem blood (**IV**) and urine samples (**III**) were collected by the forensic pathologists of the Finnish Institute for Health and Welfare (Helsinki, Finland). Individual blood (**IV**) and urine (**III**) samples from living persons were given by healthy volunteers. Pooled blood (**IV**) from living persons was acquired from the Finnish Red Cross Blood Service (Helsinki, Finland). Sheep blood (**I**) was purchased from Bio Karjalohja Oy (Karjalohja, Finland). All blood and urine samples were stored at 4ºC in preserved tubes containing 1% of sodium fluoride.

4.1.3 DERIVATIZATION REAGENTS

The derivatization reagents MBTFA (*N*-methyl-bis-trifluoroacetamide) (**V**) and MSTFA (*N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide) with 1% TMCS (trimethylchlorosilane) (**III**) were purchased from Thermo Fisher Scientific (Bellefonte, PA, USA), and TFAA (trifluoroacetic anhydride) (**IV**) was from Sigma-Aldrich (Steinheim, Germany).

4.2 SAMPLE PREPARATION

4.2.1 SOLUTIONS

Each reference standard was dissolved in methanol (**I-V**) or in a chloroform/pyridine (5:1) mixture (**V**) to obtain a 1 mg/mL stock solution. Thereafter, the stock solution was further diluted to obtain appropriate working solutions. Some of the reference standards and seized materials were analyzed directly after dissolution (**II** and **V**) or after derivatization (**V**).

4.2.2 LIQUID-LIQUID EXTRACTION (LLE)

All blood and urine samples were extracted by LLE prior to GC-NCD-APCI-QTOFMS analysis under basic conditions. Briefly, the samples were basified with Tris buffer (pH 11) in **I** and **III**, and urine samples were additionally treated with 1M NaOH. In **IV**, 5% ammonium hydroxide was used. Subsequently, samples were mixed with an appropriate extraction solvent, involving butyl acetate (**I**) or butyl chloride/ethyl acetate in volumetric ratios of 1:3 (**III**) and 3:1 (**IV**). Additionally, 0.03 g of NaCl was added to assist the transfer of analytes into the organic phase in **IV**. After centrifugation, the organic phase was collected into a conical autosampler vial for derivatization (**III-IV**) or for direct instrument analysis (**I**).

4.2.3 DERIVATIZATION

In **III**, an aliquot of the organic phase was mixed with 40 µL of MSTFA + 1 % TMCS silylation reagent and incubated for 15 min at 50 °C. In **IV**, an aliquot of the organic phase was mixed with 15 µL of TFAA acylation reagent and incubated for 15 min at 50 °C. After cooling, the mixture was neutralized with 700 µL of $NaHCO_3$, centrifuged, and the organic phase was collected for subsequent analysis. In **V**, an aliquot of the organic phase was mixed with 50 µL of MBTFA acylation reagent and incubated for 30 min at 70 °C.

4.3 METHODS

4.3.1 CONFIGURATION OF THE GC-NCD-APCI-QTOFMS PLATFORM

A 7890B Series GC System equipped with a 7693 Automatic Liquid Sampler and a split/splitless injector was coupled through a G3180B Two-Way Splitter with Makeup Gas (He) to an APCI 6540 UHD Accurate-Mass QTOF mass analyzer and a 255 Nitrogen Chemiluminescence Detector (all Agilent Technologies, Santa Clara, CA). The configuration and operating principle are briefly illustrated in Figure 4.

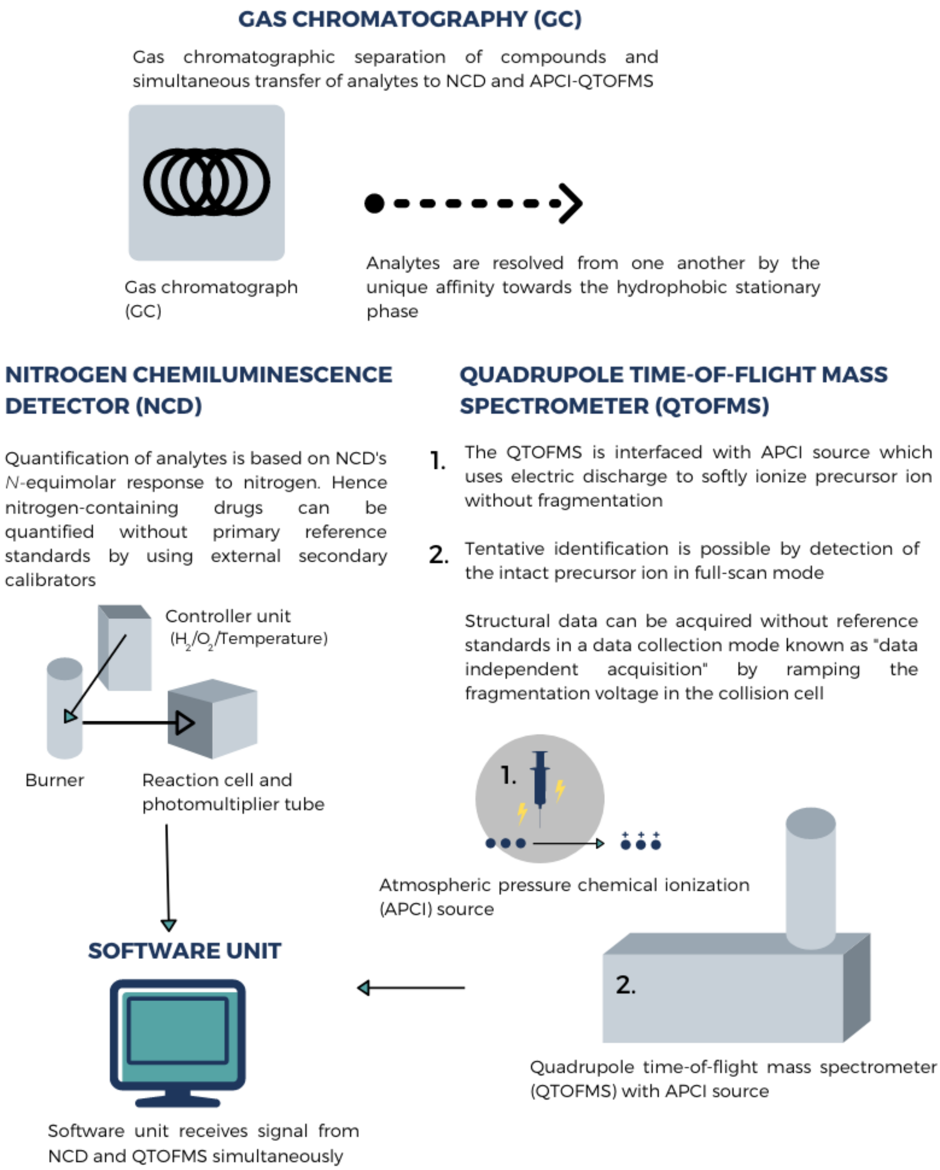


Figure 4. The main components and operating principle of the GC-NCD-APCI-QTOFMS platform.

4.3.2 GAS CHROMATOGRAPHY (GC)

The injector liner was a Single taper Ultra Inert liner with glass wool (Agilent 5190-2293). The analytical column was a DB-5MS (30 m × 0.25 mm id with 0.1 µm film) capillary column (Agilent Technologies). After the analytical column, the GC flow was divided between the NCD and the APCI ion source through a two-way splitter, using 0.55 m x 0.18 mm and 2 m x 0.18 mm uncoated deactivated fused-silica post-columns to obtain a 10:1 flow ratio, respectively. The splitter pressure was 15.8 psi and the flow ratio was calculated using the Effluent Splitter Calculator (with Makeup) (Agilent Technologies). In this concurrent detection, the NCD signal arose 0.02 minutes earlier than that of QTOFMS. In **I-IV**, the GC was operated in the pulsed splitless injection mode with an equilibrium time of 0.75 min and 50 mL/min purge flow to split vent at 0.75 min. A pulse pressure of 50 psi was applied prior to using initial head pressure of 24.9 psi. In **V**, split injection mode was used at a ratio of 10:1. The injector port temperature was 250°C and transfer line temperature 320°C. The injection volume ranged from 1 to 5 µL, depending on the application. In **I-IV,** the oven temperature was initially held at 100°C for 0.5-0.75 min and then increased by 30°C per min to 320°C, which was held for 6 min. In **V,** the oven temperature was initially held at 80°C and then increased by 30°C per min to 280°C and at the rate of 10°C per min to 320°C, which was held for 4 min.

4.3.3 QUADRUPOLE TIME-OF-FLIGHT MASS SPECTROMETRY (QTOFMS)

The QTOFMS was operated in the APCI positive ionization mode, with the drying gas (nitrogen) flow at 5.0 L/min and gas temperature at 365 °C. The current of the corona discharge needle was 1000 nA and capillary voltage 1000 V. The fragmentor voltage was 140 V (**II-V**) or 150 V (**I**) and skimmer voltage 65 V. In **I** and **II,** the mass acquisition was performed in the targeted MS/MS mode, and in **III-V**, in the all ions mode (DIA) in which collision energy ramps from 0 eV (low energy function) to 10-22 eV (high energy function) were used. The data was acquired at an *m/*z range of 50-500 with an acquisition rate of 5 spectra/s. External mass calibration was carried out using the ESI or APCI tuning mix (Agilent Technologies). The ion *m/z* 257.2475 was used for internal calibration throughout the chromatographic separation. The QTOFMS was operated in 2 GHz, Extended Dynamic Range mode. The identification criteria were as follows: the maximum mass error for precursor and product ion was set at 1-2 mDa. In **III** and **IV,** the retention time tolerance was set at 0.1 and 0.02 min, respectively. In **IV**, the intensity threshold was set at 10 000 counts for the precursor ion and 1000 counts for the product ion. The target mass of the precursor ion was based on the accurate mass of the protonated precursor ion or its acyl or trimethylsilyl derivative with an increased mass of 95.9823 or 72.0396 Da, respectively. The GC-NCD-APCI-QTOFMS spectral library used in **II** was based on averaged spectra over 12 intra-day measurements. The library scoring system algorithm used a reversed search query which included monoisotopic mass, relative abundances and isotope spacing to give a score that scaled from 0 (no match) to 100 (identical match). Data acquisition and processing were performed with the MassHunter Data Acquisition B.04.00, MassHunter Profinder B.06.00, MassHunter Personal Compound Database and Library Manager B.07.00 and MassHunter Qualitative analysis B.07.00 software (all Agilent Technologies). The GC-APCI-QTOFMS in-house library (**II**) was created using a high sample concentration (5 µg/mL) to ensure that even the polar substances could be detected. In **IV**, the relative ion response compared to a secondary calibrator was obtained by comparing the slope values with two calibration points (0.05 and 0.25 µg/mL).

4.3.4 NITROGEN CHEMILUMINESCENCE DETECTION

Pyrolysis of the analytes in the NCD was carried out at 900 °C under a hydrogen flow rate of 4 ml/min and an oxygen flow rate of 9.4 ml/min. Data from the NCD was collected at 50 Hz over the entire course of the analysis. OpenLab CDS Chemstation GC driver A.02.05.021 was used to control the GC-NCD. In quantitative estimation, a linear regression model was constructed with an appropriate external calibrator. The peak area was corrected according to the relative nitrogen content prior to applying the linear regression model. All peak areas were normalized to the peak area of the internal standard, which was either dibenzepin-d3 (**I-II**) or buspirone (**IV-V**).

4.3.5 LIQUID CHROMATOGRAPHY – CHEMILUMINESCENCE NITROGEN DETECTION (LC-CLND)

Purity estimation of seized material (**V**) was complementarily investigated by the LC-CLND technique, equipped with an ultraviolet diode array detector (UV-DAD), using caffeine as an external calibrator [124]. The LC was an 1100 series system instrument, equipped with an autosampler, a binary pump, a column oven, a 1260 infinity degasser, and a 1260 Infinity UV-DAD (all Agilent Technologies). The CLND was an Antek (PAC, Houston, TX, USA) 8060 model, which was coupled in series after UV-DAD. The LC separation was performed in a gradient mode at 40 °C, using 0.1% formic acid and methanol as mobile phase. The flow rate was 0.25 mL/min and injection volume was 10 µL. The proportion of methanol was increased from 10% to 90% over 15 min and held at 90% for 4 min. The post-time was 9 min. Caffeine calibration standards were prepared by diluting with 0.1% formic acid: methanol 90:10 (v/v) to obtain caffeine concentrations of 1.0, 2.0, 3.0, 4.0, 5.0, 10, 20, 30, 40 and 50 µg/mL which were equivalent to 2.9-144 ng of nitrogen per injection.

5 RESULTS AND DISCUSSION

5.1 GENERAL WORK-FLOW

The GC-NCD-APCI-QTOFMS platform was designed to enable the monitoring and quantification of suspect NPS with acceptable accuracy in the absence of PRS. Figure 5 summarizes the analytical goals and shows the sample preparation procedures in **I-V**.

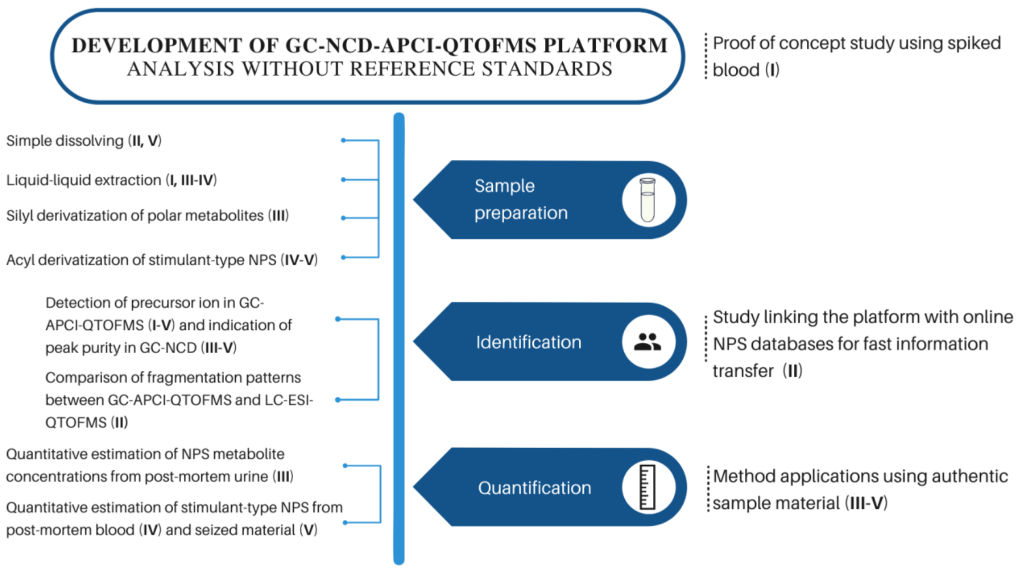


Figure 5. Summary of the topics of articles **I-V** and related sample preparation methods.

In the GC-NCD-APCI-QTOFMS workflow, a list of targeted monoisotopic masses was created based on the molecular formula of the protonated precursor ion $[M+H]^+$, taking into account the mass increase resulting from derivatization. For example, the precursor ion of pentedrone has a formula of $C_{12}H_{17}NO$ which corresponds to $C_{14}H_{16}NO_2F_3$ + H^+ after protonation and derivatization by MBTFA (Figure 6).

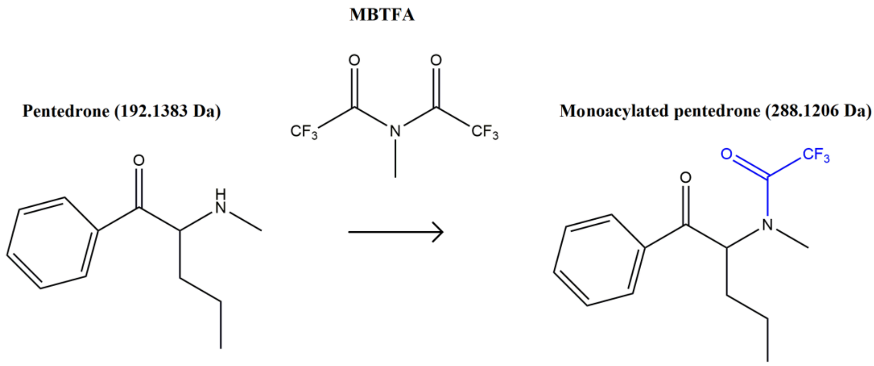


Figure 6. Acylation of pentedrone by MBTFA, resulting in mass increase from 192.1383 Da to 288.1206 Da (+95.9823 Da).

After instrumental analysis, three separate windows were monitored: 1) Extracted ion chromatogram of library/database search for tentative identification, 2) NCD chromatogram for quantification, and 3) MS/MS in DIA for detailed structural analysis or retrospective identification (Figure 7). Since separation is performed by one GC instrument, the retention time connects the information obtained from the two detectors, NCD and APCI-QTOFMS. In the split flow configuration used, the NCD retention time is always 0.02-0.03 min ahead of APCI-QTOFMS.

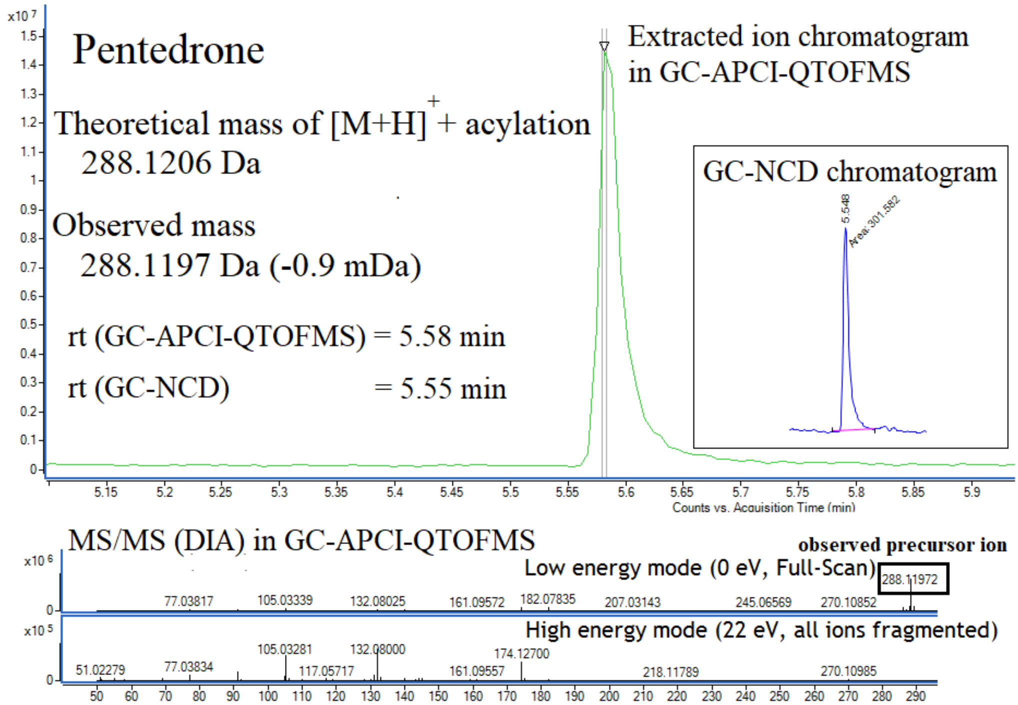


Figure 7. Visual analysis windows of GC-NCD-APCI-QTOFMS in the measurement of monoacylated pentedrone. Tentative identification was accomplished by finding the expected mass of the precursor ion in the extracted ion chromatogram (upper left). Constant acquisition of MS/MS spectra in DIA (below) at specific collision energies allowed the acquisition of structural information without pre-experiment targeting. Lastly, the qualitative MS results were combined with the GC-NCD quantification results (upper right) using the matching retention times.

5.2 DERIVATIZATION

Derivatization was used to increase the volatility of polar substances and prevent their decomposition and adsorption into the GC system [129, 130]. To ensure both qualitative and quantitative accuracy, the urinary α-PVP metabolites were silylated (**III**), and the parent stimulant-type NPS were acylated (**IV-V**). Trimethylsilylation by MSTFA produced a consistent peak shape for the hydroxyl (M1) and amino (M5) metabolites of α-PVP, which initially showed distorted peak shapes. However, MSTFA and trimethylsilyl (TMS) derivatives are sensitive to residual moisture that may lead to undesirable hydrolysis of reaction products. Hence, acylation was preferred in cases where only primary and secondary amines were targeted. TFAA was used in **IV**, but in **V** it was replaced with MBTFA to adhere to the adapted sample preparation procedure for seized powders [131]. Moreover, unlike TFAA, derivatization by MBTFA is more straightforward since its by-product *N*-methyltrifluoroacetamide is a volatile and neutral compound which can be readily injected into the GC column [130]. General equations for the derivatization reactions used in this study are shown below:

Trimethylsilylation of alcohols and amines with MSTFA

$$R\text{-}OH + (CH_3)_3Si\text{-}X \rightarrow R\text{-}O\text{-}Si(CH_3)_3 + HX$$
$$R\text{-}NH + (CH_3)_3Si\text{-}X \rightarrow R\text{-}N\text{-}Si(CH_3)_3 + HX$$

Acylation of amines with MBTFA or TFAA

$$R\text{-}NH + COCF_3\text{-}X \rightarrow R\text{-}N\text{-}COCF_3 + HX$$

Before quantitative analysis, the derivatization efficiency was studied by searching the extracted ion chromatograms for the underivatized precursor ion together with the respective derivatization product. Consequently, in addition to the two α-PVP metabolites that were successfully trimethylsilylated, 21 primary amines and 48 secondary amines were acylated with TFAA in the blood (**IV**) or by MBTFA in the powdered material (**V**). From these amines, only methoxetamine and 5-MeO-MIPT were not fully derivatized. In case of methoxetamine, it is likely that steric hindrance prevented the reaction from going to completion. Following this observation, incomplete derivatization of methoxetamine has also been reported after acetylation [132]. The secondary amino group of 5-MeO-MIPT is a part of indole moiety and difficult to derivatize with MBTFA. Nevertheless, both methoxetamine and 5-MeO-MIPT retained an excellent peak shape after reanalysis in the absence of the acylation reagent.

5.3 IDENTIFICATION

5.3.1 ACCURATE MASS MEASUREMENT OF THE PRECURSOR ION

In-source fragmentation of the precursor ion may obscure the process of tentative identification. This problem is especially relevant in GC, where EI is commonly used as an ion source. However, previous studies on endogenous metabolites, pesticides and organic pollutants have shown that GC with an APCI source retains the intact precursor ion, and consequently GC could be coupled with HRMS for accurate tentative identification similarly to LC-ESI-HRMS techniques [133-135]. In the evaluation of the present GC-NCD-APCI-QTOFMS platform, one important objective was to verify these observations before carrying out subsequent quantitative studies in **I** and **III-V**. In all of these articles, the maximum mass error was set at 1-2 mDa, which enabled successful tentative identification of all the studied substances. However, accurate mass alone is still prone to false-positive identification when isomeric substances with the same mass are encountered. A more comprehensive analysis of the acquired spectral data could reduce the number of false-positive identifications. Such approaches include isotopic pattern matching [136], limitation of the number of elements

[136], retention time prediction [137], and *in silico* fragmentation algorithms [138-141]. However, these strategies were beyond the scope of this research. Furthermore, all NPS except the α-PVP metabolites in post-mortem urine (**III**) were previously confirmed by analysis with PRS.

5.3.2 SPECTRAL LIBRARY AND DATABASE (II)

Rapid detection procedures and toxicological risk assessment are needed to mitigate the harms of NPS [73, 81]. Online spectral libraries and databases increase the confidence of identification, because they contain useful information on the molecular formulae and fragmentation pattern [86]. However, these data have been primarily collected by using LC-ESI-HRMS techniques instead of GC-APCI-HRMS. Lack of available online spectral libraries and databases specific to GC-APCI limits inter-laboratory collaboration within the scope of GC-NCD-APCI-QTOMFS. Article **II** aims to prove that online NPS databases created by LC-ESI-HRMS could be useful instead.

Spectral libraries and databases offer an indispensable tool for screening of unknown compounds without PRS. A widely used library for GC-MS is the NIST/EPA/NIH Mass Spectral Library. Impressively, the 2020 version contains 350 643 low-resolution EI spectra from 306 869 chemical compounds [142]. On the other hand, GC libraries supporting the APCI source are unavailable despite the advantage of the combination in detecting the intact precursor ion. One explanation may be that construction, standardization and maintenance of high-quality spectral libraries and databases is not practical for less widespread techniques. Therefore, *in silico* generated databases could be used for tentative identification. A promising workflow for GC-APCI-QTOFMS was developed by Ruttkies et al. (2015). They used structures from chemical databases (KEGG and PubChem) to perform *in silico* derivatization prior to *in silico* fragmentation [143].

According to the research hypothesis expressed in **II**, MS/MS spectra should be similar across different QTOFMS analyzers, regardless of the type of hyphened chromatography or ion source, assuming that the intact precursor ion is detected in sufficient abundance to produce consistent spectra. Hence the main difference between varying techniques lies in the ability to generate a sufficient abundance of intact precursor ions that will eventually reach the collision cell. To prove this hypothesis, the performance of a commercially available LC-ESI-HRMS library was compared to an in-house spectral library that was created by GC-NCD-APCI-QTOFMS, using the Agilent MassHunter Personal Compound Database and Library Manager software. In Figure 8, the spectral data for 29 drugs of abuse was used to evaluate the similarity of these two libraries based on the following four criteria; 1) “Major fragments”: detection of five most abundant fragments in both libraries, 2) “Fragmentation pattern”: absence of unique fragmentation products, 3) “Library score”: manufacturer’s scoring system for library identification, and 4) “Fragment ion ratios”: similar ion ratios for three of the most abundant fragments.

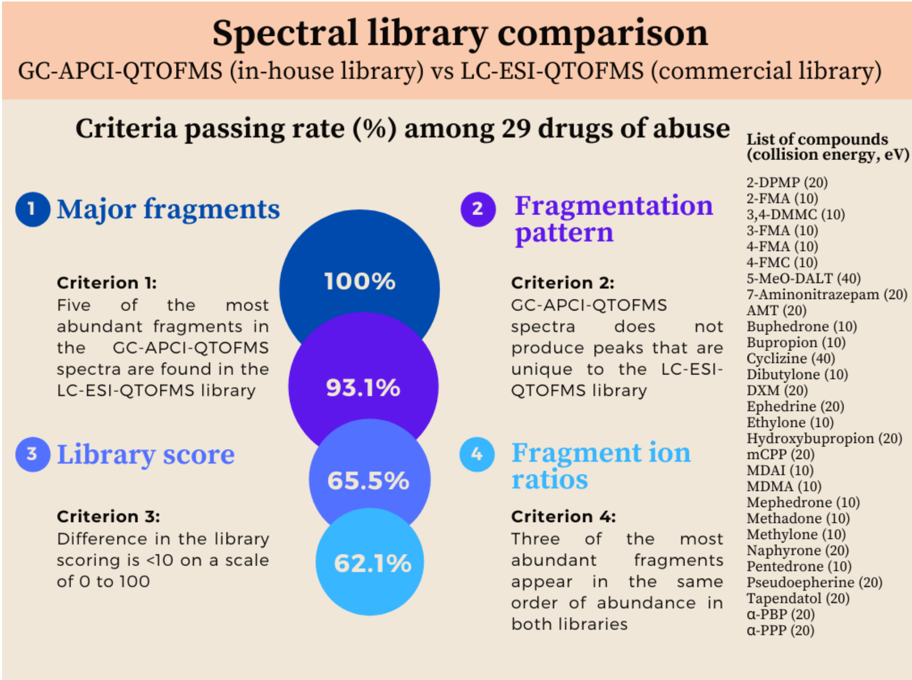


Figure 8. Comparison of GC-APCI-QTOFMS and LC-ESI-QTOMFS libraries containing 29 drugs of abuse using four evaluation criteria. The figure was reconstructed using MS/MS data from **II**. On the right panel, the substances are listed together with their optimal collision energies (in brackets) used in the study.

In this evaluation, the detection of major fragments (criterion 1) and the dissimilarities of fragmentation pattern (criterion 2) are both showing high acceptance rates for these criteria, with 29/29 (100 %) of the substances passing the criterion 1 and 27/29 (93.1 %) passing the criterion 2. These two criteria describe similarities between the two libraries qualitatively, emphasizing the detection of peaks with the same identity. On the other hand, library score (criterion 3) and fragment ion ratio (criterion 4) are largely influenced by the fragment ions' relative abundance, and consequently the passing rate by these criteria was much lower, 65.5 and 62.1 %, respectively. These results suggest that identification by GC-NCD-APCI-QTOFMS should be carried out using an HRMS database with diagnostic fragments instead of full mass spectral libraries. It is likely that identification by mass spectral libraries, in this case, is more prone to errors because the degree of molecular fragmentation and ionization is difficult to optimize across different vendor platforms, ion sources and method variables.

5.4 QUANTIFICATION

Quantification without PRS by GC-NCD using an external nitrogen-containing secondary calibrator was accomplished in four separate studies: investigation of NPS metabolites in urine (**III**), quantification of NPS in blood (**I, IV**), and purity estimation of NPS in seized powdery material (**V**).

5.4.1 MATRIX INTERFERENCES

Co-elution of a target analyte with matrix interferences can potentially result in an overestimation of analyte concentration in quantitative analysis by NCD, and therefore the blank matrix background should be carefully studied in both NCD and QTOFMS chromatograms. Consequently, subsequent examination of full-scan QTOFMS spectra allowed detection of possible interference in such occasions, where co-elution was not visible in the NCD chromatogram. Interferences deriving from endogenous substances were mainly observed in the NCD chromatograms from blood and urine samples. As expected, sample derivatization increased the number of interfering peaks, because it improved the volatility and detectability of minor nitrogenous matrix components. In general, the likelihood of interferences detected decreased in the following order based on the sample type and preparation: derivatized urine > derivatized blood > urine > blood > derivatized powdery material > powdery material. Fortunately, a large proportion of the components of background noise, including residues from the buffer and derivatization reagent, were eluting much earlier than typical drug molecules. Some of the quantitative NCD results were rejected because of proven interference based on the corresponding QTOFMS spectrum. These cases were always manually evaluated before quantitative analysis. In such an evaluation, the NCD chromatogram was inspected for peak shape abnormalities, and the major ions in the QTOFMS spectrum should only belong to the target analytes or known matrix components not interfering with the analysis of blank material.

5.4.2 QUANTIFICATION OF α-PVP METABOLITES IN URINE (III)

An earlier publication by Tyrkkö et al. (2013) identified seven candidate structures for the urinary metabolites of α-PVP, based on the findings from *in vitro* studies or *in silico* analysis [139]. The accurate mass of each of these seven metabolites was targeted in post-mortem human urine samples previously found positive for α-PVP, and all the seven metabolites were found by GC-APCI-QTOFMS (Figure 9). Subsequent to these initial findings, the PRS for the metabolites M1, M3, and M5 were purchased from a commercial vendor for quantitative confirmation analysis.

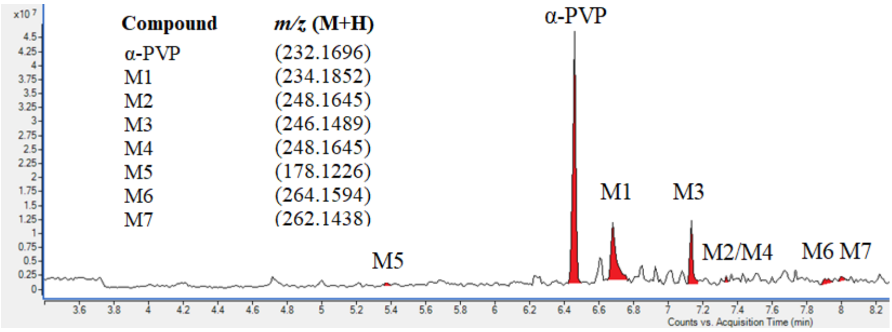


Figure 9. GC-APCI-QTOFMS chromatogram from a post-mortem urine sample showing peaks with a matching accurate mass (± 1mDa) for α-PVP and its metabolites.

Quantitative method validation experiments with spiked specimens showed that the between-day bias and imprecision were regularly below 27% and < 13% (CV), respectively, when quantification by GC-NCD was performed using the parent drug α-PVP as a secondary calibrator. However, using a similar strategy in APCI-QTOFMS, poor quantitative accuracy was obtained, especially in the determination of the hydroxyl group - containing metabolite M1. By APCI-QTOFMS, the bias for M1 in the low-concentration point (0.25 µg/mL) was 60% and in the high-concentration point (1 µg/mL) 58%, as opposed to the better results by GC-NCD (24% and 25%, respectively). Although only three substances were tested, this results was consistent with the earlier observations on secondary calibrator -based quantification attempts by MS, suffering from structure specific ion response [8, 98].

Table 2 shows that nearly all of the 20 post-mortem urine samples included in the study were found positive for the target metabolites, based on the monitoring of the respective protonated precursor ions with high mass accuracy. The metabolites M1 and M5 reacted successfully with MSTFA forming TMS derivatives, as proven by a mass increase of 72.0396 Da. However, some of the quantitative data could not be properly acquired due to the limited analytical sensitivity and matrix interferences.

Table 2. Tentative identification and quantitative estimation of α-PVP and its metabolites in 20 post-mortem urine samples by GC-NCD-APCI-QTOFMS. Successful quantitative estimation was based on three prerequisites; 1) the detection of protonated molecule in APCI-QTOFMS spectrum, 2) sufficient sensitivity of GC-NCD and 3) the absence of co-eluting nitrogenous compounds in GC-NCD.

Substance	Rate of detection in APCI-QTOFMS	Above LOQ in GC-NCD	Clear of interference	Mean (range) concentration by GC-NCD (µg/mL) [a]	Mean bias (%)	Mean CV (%)
α-PVP	100% (20/20)	65% (13/20)	92% (12/13)	2.28 (0.3-8.04)	5.8	13
M1	100% (20/20)	70% (14/20)	79% (11/14)	1.50 (0.18-5.18) [b]	26.9	8
M3	95% (19/20)	37% (7/19)	29% (2/7)	0.54 (0.38-0.7)	9.9	2
M5	85% (17/20)	0% (0/17)	-	-	-	-

[a] Excluding samples below the limit of quantification (LOQ) level (16 pg/N, corresponding to approximately 0.25 µg/mL of derivatized M1).
[b] One result at 0.18 µg/mL was extrapolated below the lowest calibration point (0.25 µg/mL).

5.4.3 QUANTIFICATION OF STIMULANTS IN BLOOD (I, IV)

Introduction of the proof-of-principle for the GC-NCD-APCI-QTOFMS platform was presented in **I,** using sheep blood spiked with bupropion, 2-DPMP, mephedrone, methylone and naphyrone. In that study, the role of the extraction recovery was ignored by spiking the blood post-extraction. Article **IV** elaborated the concept in a more realistic experimental setting: 38 illicit psychostimulants spiked in blood were analyzed taking advance of the control of extraction recovery by secondary calibrators. Compared to urine, blood samples showed considerably less matrix interferences in the GC-NCD chromatogram. Consequently, the lack of interferences and the possibility to use a higher injection volume (5µL) improved the sensitivity of GC-NCD, resulting in a generic limit of quantification (LOQ) of 0.05 µg/mL.

The 38 NPS stimulants were chosen for the method development experiments based on the availability of the corresponding PRS in the laboratory. Fortunately, this starting point resulted in a structurally diverse group of stimulants to be tested. The mean between-day bias and imprecision at the LOQ level (0.05 µg/mL) by GC-NCD were as good as 19% and 16% (CV), respectively. The highest bias of 44.3% was obtained with PCP (phencyclidine). In the developed method, the matrix interferences prevented reliable quantification of MBDB and, additionally, prevented quantification of dibutylone and methylphenidate at the LOQ level. Hence, quantification of the interfered targets might require method adjustment, such as changing the GC column. As for APCI-QTOFMS, the mean bias was as high as 60%, while the highest bias was found with naphyrone (186%). Comparison of the quantitative performance between GC-NCD and APCI-QTOFMS is illustrated in Figure 10. The figure shows that a bias higher than 30%, as well as the extreme values, were much more frequent with APCI-QTOFMS (in 24 cases, bias of 31-186%) than with GC-NCD (in five cases, bias of 31-43%).

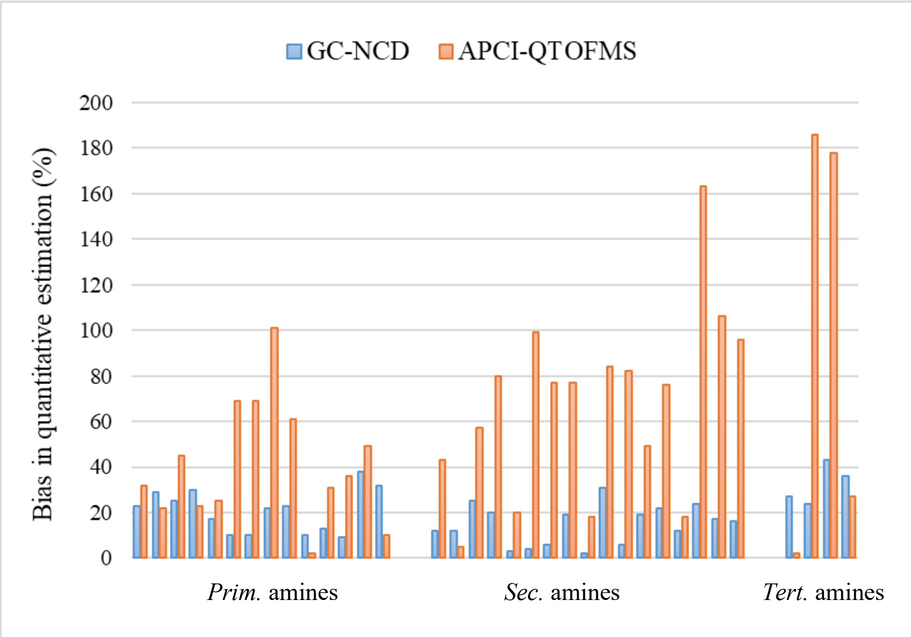


Figure 10. Measurement bias in the quantitative estimation of 35 stimulants spiked in blood by using a secondary calibrator. Three substances (MBDB, dibutylone and methylphenidate) were excluded due to matrix interferences in GC-NCD. The following secondary calibrators were used: amphetamine for *prim.* amines, MDMA for *sec.* amines, and MDPV for *tert.* amines. Blue columns represent actual quantitative measurements by GC-NCD at LOQ (0.05 µg/mL), and red columns represent the deviation of the ion response slope between the target analyte and secondary calibrator in APCI-QTOFMS.

5.4.4 QUANTIFICATION OF STIMULANTS IN SEIZED POWDERS (V)

Analysis of powdery drugs without PRS using the *N*-equimolar principle of LC-CLND was pioneered by Laks et al. (2004) [9]. However, prior to this thesis, a similar approach has not been exploited in the GC-NCD environment or in conjunction with HRMS, altough the approach would enable the examination of peak identity and the detection of possible co-eluting interferences. In **V**, a method was developed and validated for 28 stimulant drugs using a sample preparation method amenable to GC analysis: the drugs were dissolved in chloroform/pyridine (5:1) solution, acylated with MBTFA, and measured over five separate experiments. The mean bias was as low as 12%, with only cocaine showing a bias higher than 30% (36.2%). In all samples, the imprecision was always below 10% (CV), with a mean value of 5.5%. Subsequently, 42 seized powders of unknown purity were quantified by using both the newly developed GC-NCD method and an established LC-CLND method [124] for comparison. The mean purity value difference between the methods was on average 8.1 percentage units (range 0.4-26.7 percentage units). Although the results were mostly consistent, it is likely that some of the larger discrepancies arose from solubility differences (in LC-CLND methanol was used). As compared to the LC-CLND method by Laks et al. (2004) [9], in GC-NCD the sample preparation and data interpretation stages are less straightforward, since derivatization of polar analytes is required.

5.4.5 COMPARISON OF DEVELOPED METHODS

The results from method validation experiments show that the quantitative performance remained adequately consistent despite the fact that very different sample materials were tested. This can be observed in Figure 11, which shows the relationship of the quantitative accuracy and precision across the GC-NCD methods using the grand mean values (the mean of five or six between-day repeats across all concentration data points) from a total of 63 individual analytes. Seized powders (**V**) were generally measured with a slightly better precision compared to extracted blood samples (**IV**) (mean CV 7.1% vs. mean CV 11.6%, respectively), presumably due to a simpler sample preparation procedure for the powdered samples. Accuracy remained consistent throughout the used concentration range in both studies: 92.5-97.8% for extracted blood samples with a concentration range of 0.05–1.25 µg/mL (**IV**), and 87.9-90.9% for seized powders with a concentration range of 40-200 µg/mL (**V**). Regrettably, **I** and **III** contained only five and three separate analytes, respectively, and therefore the interpretation is only directive. The largest measured underestimation was observed with naphyrone in blood (bias -59.0%), and the largest overestimation with cocaine in a seized powdery sample (bias +45.0%). The best sensitivity (LOD 0.05 µg/mL) was reached in **IV**, mainly due to the fact that the highest possible injection volume (5 µL) was used.

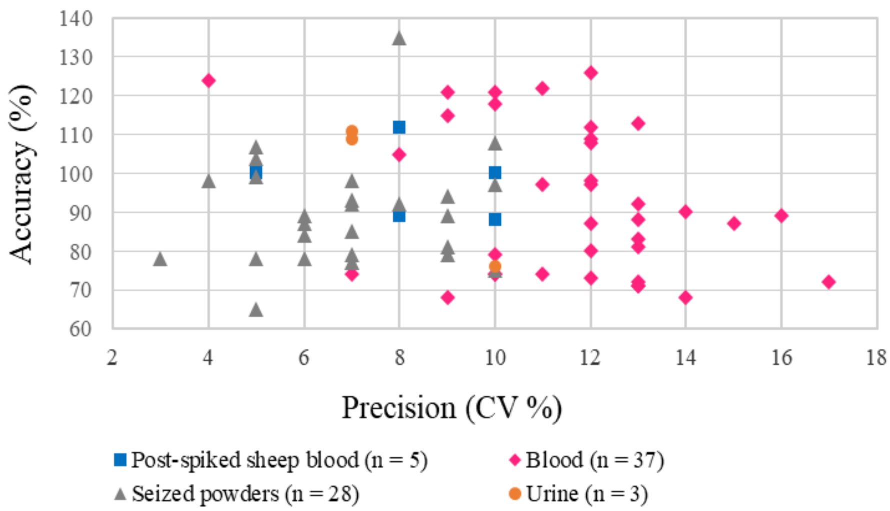


Figure 11. Comparison of quantitative estimations from the GC-NCD-APCI-QTOFMS studies of this thesis. The data points are the grand mean values obtained from all between-day accuracy and precision values from five or six separate experiments across the whole concentration range of the method: seized powders, 40-200 µg/mL (**V**), urine, 0.25-1.00 µg/mL (**III**), blood, 0.05-1.25 µg/mL (**IV**), and post-spiked sheep blood, 0.17-1.70 µg/mL (**I**).

6 GENERAL DISCUSSION

It is well known that laboratory analysis of NPS is obstructed by the lack of certified PRS [81]. Therefore, the goal of early detection and toxicological assessment, followed by effective allocation of resources, should be met with a strategy that is fit for the analytical problem. In this study, a new analytical platform is introduced to combat the problem of lacking PRS and to provide means for instant analysis of NPS.

Already established, fast detection of NPS without PRS can be accomplished with an LC-ESI-HRMS instrument, typically a QTOFMS or an Orbitrap mass analyzer [97], with use of accurate mass –based spectral libraries or databases. However, until now, the acquisition of quantitative data has been ignored mainly because of the technical challenges of MS quantification (e.g., structure-dependent ion response), which often lead to a gross measurement bias if the corresponding PRS are not used [7, 8, 98]. Consequently, an uncertainty factor has been used for the establishment of threshold values for toxicological decision-making to avoid possible under- or overestimations in quantitative analysis. In essence, the uncertainty factor is based on the theoretical probability distribution of measurement bias, and it has already been proposed for specific applications, such as for the toxicological evaluation of drug metabolites [98] and for the characterization of extractables and leachables [144]. However, such an approach has only limited usability if the margin of error remains high. Instead, a viable strategy to improve quantitative estimation is to utilize a universal detector that possesses a uniform response regardless of the structural composition of the substance. The GC-NCD-APCI-QTOFMS platform developed in this thesis enables simultaneous quantitative analysis based on the NCD detector's *N*-equimolar principle with use of secondary calibrators, which makes the technique almost universally valid in drug analysis.

The number of publications applying NCD/CLND has been steadily growing across various disciplines, and many sample types have been examined, including fuels [145-147], bio-oils [148], water [149], plant extracts [119, 150], house dust [151], soil [152], food [153, 154], whisky [155], latex products [156], urban aerosol [157], drugs [9, 124, 158, 159], organic compounds [110, 122] and blood [126, 128]. Interestingly, a multidetector approach has been used to create synergy in many analytical applications. For instance, Ochiai et al. (2012) utilized sulfur chemiluminescence detector (SCD) mainly to characterize sulfur substances in whisky, but they complemented their analysis with NCD to investigate the presence of nitrogen-containing substances. Jiang et al. (2015) introduced an LC-MS/UV/CAD/CLND platform, in which the quantification of pharmaceuticals was carried out by both CAD and CLND. In effect, these two detectors provided complementary information, since CLND is limited to nitrogen-containing compounds and CAD is not suited for the analysis of volatile substances that do not form particles [158]. Although the profiling and total quantification of the nitrogen containing fraction has commonly been performed by NCD, the detector's unique advantage of *N*-equimolar quantification without PRS has rarely been applied in published studies. This gap in knowledge may be due to the fact that specific substance identification has been incomplete due to insufficient identification capabilities.

The combination of three main technical advances previously presented in analytical instrumentation has allowed the successful development of the present GC-NCD-APCI-QTOFMS platform for NPS analysis. Firstly, the possibility to acquire full-scan spectra for tentative identification by HRMS; secondly, the possibility to detect an unfragmented protonated molecule by using the APCI ion source, instead of the highly fragmenting EI source; thirdly, the possibility to apply NCD in quantitative analysis based on its *N*-equimolar principle. GC-APCI has only recently become available due to a better commercial availability of the technology. It is vital to emphasize that the pioneers of GC-APCI-QTOFMS instrumentation [133-135] have encouraged the conceptualization of the present GC-NCD-APCI-QTOFMS platform.

In comparison to some of the older studies utilizing stand-alone LC-CLND, such as the one by Ojanperä et al. (2007) [128], the GC-NCD peak width obtained in this thesis was much narrower for similar amounts of drugs in blood after LLE. This is due to the fact the LC-CLND instrument brand available (Antek 8060 CLND) did not support the higher resolution and narrower peaks associated with modern ultra-high performance LC (UHPLC). Consequently, the current GC-NCD version was capable of producing a better chromatographic resolution than was obtained in the older studies, which is invaluable in the analysis of complex mixtures.

One of the most critical limitations of GC-NCD-APCI-QTOFMS is the sensitivity of NCD, which in the present studies has been approximately 0.05 µg/mL in blood analysis (**IV**), slightly depending on the relative nitrogen content of each drug. As such, the analytical platform is ideal for stimulant-type NPS, which are present in biological samples at higher concentrations than representatives of some other drug classes, such as fentanyls and synthetic cannabinoids [69]. One possibility to enhance the sensitivity would be to include a concentration step, involving the evaporation of extraction solvent, in the sample preparation step. However, evaporation to dryness was deliberately avoided here because of potential losses of volatile substances. In case of the α-PVP metabolites in **III**, the concentrations of the less abundant metabolites M3 and M5 were often below the LOQ in the post-mortem urine samples studied. The sensitivity of APCI-QTOFMS was always better than that of NCD for all of the studied NPS, which is why the LOD obtained was significantly better than the LOQ despite the fact that the sensitivity of APCI-QTOFMS was compromised by the uneven distribution of GC flow. In this configuration, only 1/10th of the injected flow was directed to APCI/-QTOFMS.

Interestingly, the sensitivity of APCI-QTOFMS could be further improved by an infusion of water to the APCI source, which reportedly enhanced the signal intensity and reproducibility of protonated precursor ions [160]. The sensitivity may also be improved by derivatization, which in most cases enhances ionization efficiency in MS [161]. Although the selectivity of NCD was reasonable in all studies, it is possible that some future applications to the analysis of substances with concentrations close to the LOQ level might encounter problems with the signal to noise ratio. In such a case, the possibility to use two-dimensional GC (GCxGC) should be considered to achieve better selectivity and sensitivity [162].

The introduction of a derivatization step causes potential uncertainties for analytics. For instance, the application of heat during derivatization might lead to unintentional evaporation of some analytes or the derivatization reaction could be incomplete. For this reason, following derivatization a search must be conducted against all possible products, with or without adducts, for tentative identification. Additional complexity arises in data analysis, because existing spectral libraries containing also derivatized substances for soft ionization HRMS are uncommon. Clearly, LC based instrumentation has certain advantages over GC based instrumention, such as a wider range of analytes that can be measured without the need for derivatization.

In this thesis, suspect screening was solely based on the accurate mass measurement by APCI-QTOFMS. This is generally considered insufficient for unambigious substance identification, the latter requiring additional information that could be mainly obtained from use of PRS. Attempts to improve tentative identification by other measures, such as by using isotopic patterns, *in silico* prediction of DIA fragments, or retention time and ion mobility drift times, should be elaborated in future applications. In the past, some concerns were raised by Pacchiorotta et al. (2013) about the lack of online resources for spectral libraries for GC-APCI, unlike for GC-EI or LC-ESI [163]. However, the results for 29 structurally diverse drugs (mostly NPS) in **II** show that the MS/MS spectra obtained by GC-APCI-QTOFMS are similar to those included in commercial LC-ESI-QTOFMS libraries. The results further suggest that the use of reference databases relying on precursor and major fragment ions is preferable to the use of whole spectrum libraries because of the difficulty of obtaining sufficiently matching ion ratios between instruments.

The results published for urine (**III**), blood (**IV**) and seized powders (**V**) show that the expected mean bias for quantitative analysis without PRS in extracted or dissolved samples is approximately 10%, and all substances in these studies could be measured below a two-fold error. This is a significant improvement over the semi-quantitative analysis by MS, in which the estimation of accuracy is much more unpredictable and even up to a 71-fold error in quantitative estimation has been reported [8]. In **III** and **IV**, it was demonstrated that GC-NCD-APCI-QTOFMS could be used to reveal concentrations of NPS and their metabolites while performing a drug screen. It can be anticipated that some of the most potential NPS research applications by the present approach are within retrospective drug analysis, as retrospective quantification of practically any nitrogen-containing peak is possible. Of course, in this type of application, a secondary calibrator similar to the analyte should be used. Another interesting application, which is also extremely difficult to conduct without PRS, is the measurement of metabolite to parent drug concentration ratios of NPS. Such information could help identify potential cases of poisoning and reveal individual differences caused by the pharmacogenetic variation or modulation of metabolic enzymes.

Overall, the possibility to acquire quantitative information without PRS opens doors for future research not only in forensic science but also in other disciplines outside of forensics, such as metabolomics, environmental testing and discovery of biomarkers. As a final remark, the utility of the presented platform was designed for fast tentative analysis, and therefore it does not exclude the necessity of using certified PRS for the final confirmation of initial measurements. For now, there are no officially accepted criteria for the acceptance of drug analysis results that are obtained without PRS in forensic sciences.

7 CONCLUSIONS

Determination of the concentration levels of drugs is one of the important tasks in evidence-based forensic science and, especially related to the topic of this dissertation, in the estimation of drug use and toxicity in the cause-of-death investigation and in the purity estimation of seized drugs for juridical decision-making. Prior to this thesis, rapid analysis of emerging NPS has been difficult to carry out, because conventional MS techniques are almost exclusively ruled out due to the poor availability of the necessary PRS. The developed GC-NCD-APCI-QTOFMS platform, which was first introduced in **I,** was designed to meet this problem by enabling the identification and quantification of NPS from biological samples and seized material, without immediate access to PRS.

The main concept behind the GC-NCD-APCI-QTOFMS platform was to combine the HRMS and NCD detection techniques following GC separation into one easily manageable entity that allows tentative identification based on the high mass accuracy of HRMS and quantitative evaluation based on the *N*-equimolar principle of NCD. It was proved in **II** that identification by GC-APCI-QTOFMS can be based on external spectral libraries and databases produced by conventional LC-ESI-MS/MS, which further supports the early detection of NPS using information gathered by forensic collaborators.

In **III-V**, quantitative estimation was carried out using carefully chosen external nitrogen-containing secondary calibrators. Analysis of NPS in authentic forensic material, such as seized powders (**V**), post-mortem blood (**IV**) and urine (**III**), was carried out with a mean accuracy of 91.7% (range 59.0-145.0%) and imprecision of 9.5% (CV), based on the data that was obtained from 28 NPS in seized powders, 37 NPS in post-mortem blood and three NPS metabolites in post-mortem urine. The fact that all individual measurements were performed with less than a 2-fold error, the mean bias being less than 10% in complex biological matrices, is a significant improvement over semi-quantitative MS measurements, showing up to >70-fold error even with dissolved powders [8]. However, it is noteworthy that the relatively low sensitivity of GC-NCD and the necessity to derivatize polar compounds for GC might limit some of the future applications.

In conclusion, the GC-NCD-APCI-QTOFMS platform allows rapid generic analysis of stimulant-type NPS without PRS and without method parameter adjustment for individual analytes. In comparison to other techniques that do not necessarily require PRS, such as LC-nanospray mass spectrometry or quantitative NMR, the present approach stands out due to its facile operation and the low cost of the NCD detector.

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9 REFERENCES

1. United Nations (1961) Single convention on narcotic drugs. Available at: https://www.unodc.org/pdf/convention_1961_en.pdf (accessed 25.8.2020).

2. United Nations (1971) Convention on psychotropic substances. Available at: https://www.unodc.org/pdf/convention_1971_en.pdf (accessed 25.8.2020).

3. United Nations Office on Drugs and Crime (2020) Early warning advisory on new psycohactive substances. Available at: https://www.unodc.org/LSS/Page/NPS (accessed 10.5.2020).

4. European Monitoring Centre for Drugs and Drug Addiction and Europol (2019), EU Drug Markets Report 2019, Publications Office of the European Union, Luxembourg. Available at: http://www.emcdda.europa.eu/publications/joint-publications/eu-drug-markets-report-2019 (accessed 15.1.2020).

5. Sundström M, Pelander A, Angerer V, Hutter M, Kneisel S, Ojanperä I (2013) A high-sensitivity ultra-high performance liquid chromatography/high-resolution time-of-flight mass spectrometry (UHPLC-HR-TOFMS) method for screening synthetic cannabinoids and other drugs of abuse in urine. *Anal Bioanal Chem.* **405**:8463-8474.

6. Kinyua J, Negreira N, Ibanez M, Bijlsma L, Hernandez F, Covaci A, Nuijs A (2015) A data-independent acquisition workflow for qualitative screening of new psychoactive substances in biological samples. *Anal Bioanal Chem.* **407**:8773-8785.

7. Cech NB, Enke CG (2001) Practical implications of some recent studies in electrospray ionization fundamentals. *Mass Spectrom Rev.* **20**:362-387.

8. Hatsis P, Waters NJ, Argikar UA (2017) Implications for Metabolite Quantification by Mass Spectrometry in the Absence of Authentic Standards. *Drug Metab Dispos.* **45**:492-496.

9. Laks S, Pelander A, Vuori E, Ali-Tolppa E, Sippola E, Ojanperä I (2004) Analysis of street drugs in seized material without primary reference standards. *Anal Chem.* **76**:7375-7379.

10. Crocq M (2007) Historical and cultural aspects of man's relationship with addictive drugs. *Dialogues Clin Neurosci.* **9**:355-361.

11. Official Statistics of Finland (OSF): Causes of death (2018), Appendix table 4. Drug-related mortality 2000 to 2018. Helsinki: Statistics Finland. Available at: http://www.stat.fi/til/ksyyt/2018/ksyyt_2018_2019-12-16_tau_006_en.html (accessed 25.4.2020).

12. International Programme on Chemical Safety & Organisation for Economic Co-operation and Development. (2004). IPCS risk assessment terminology. World Health Organization. Available at: https://apps.who.int/iris/handle/10665/42908 (accessed 24.6.2020).

13. Palleria C, Di Paolo A, Giofrè C, Caglioti C, Leuzzi G, Siniscalchi A, Sarro G, Gallelli L (2013) Pharmacokinetic drug-drug interaction and their implication in clinical management. *J Res Med Sci.* **18**:600-609.

14. Krewski D, Andersen ME, Tyshenko MG, Krishnan K, Hartung T, Boekelheide K, WamBaugh JF, Jones D, Whelan M, Thomas R, Yauk C, Barton-Maclaren T, Cote I (2020) Toxicity testing in the 21st century: progress in the past decade and future perspectives. *Arch Toxicol.* **94**:1-58.

15. Ellis CR, Racz R, Kruhlak NL, Kim MT, Hawkins EG, Strauss DG, Stavitskaya L (2019) Assessing the structural and pharmacological similarity of newly identified drugs of abuse to controlled substances using public health assessment via structural evaluation. *Clin Pharmacol Ther.* **106**:116-122.

16. Limban C, Nuţă DC, Chiriţă C, Negreș S, Arsene AL, Goumenou M, Karakitsios S, Tsatsakis A, Sarigiannis D (2018) The use of structural alerts to avoid the toxicity of pharmaceuticals. *Toxicol Rep.* **5**:943-953.

17. Deichmann WB, Henschler D, Holmstedt B, Keil G (1986) What is there that is not poison? A study of the Third Defense by Paracelsus. *Arch Toxicol.* **58**:207-213.

18. Muller PY, Milton MN (2012) The determination and interpretation of the therapeutic index in drug development. *Nat Rev Drug Discov.* **11**:751-761.

19. Hiemke C (2019) Concentration-effect relationships of psychoactive drugs and the problem to calculate therapeutic reference ranges. *Ther Drug Monit.* **41**:174-179.

20. Basalingappa S, Sharma A, Amarnath S (2015) Basic concepts of therapeutic drug monitoring. *Intl J Curr Pharm Rev Res.* **5**:70-75.

21. Singh DK, Sharma S, Thakur A, Kumar S, Singh S (2019) Pharmaceutical analysis | drug purity determination. Encyclopedia of analytical science. 188-199.

22. Andrade EL, Bento AF, Cavalli J, Oliveira SK, Schwanke RC, Siqueira JM, Calixto JB (2016) Non-clinical studies in the process of new drug development - Part II: Good laboratory practice, metabolism, pharmacokinetics, safety and dose translation to clinical studies. *Braz J Med Biol Res.* **49**:e5646.

23. Food and Drug Administration (2017) Clinical dug interaction studies — study design, data analysis, and clinical implications. Guidance for Industry.Available at: https://www.fda.gov/drugs/drug-interactions-labeling/drug-interactions-relevant-regulatory-guidance-and-policy-documents (accessed 6.1.2021).

24. Lapachinske SF, Okai GG, dos Santos A, de Bairros AV, Yonamine M (2015) Analysis of cocaine and its adulterants in drugs for international trafficking seized by the Brazilian Federal Police. *Forensic Sci Int.* **247**:48-53.

25. European Monitoring Centre for Drugs and Drugs Addiction (2003) The role of the quantity in the prosecution of drug offences. Available at: http://www.emcdda.europa.eu/system/files/publications/10503/Quantities.pdf (accessed 6.1.2020).

26. Wurst FM, Metzger J (2002) The ethanol conjugate ethyl glucuronide is a useful marker of recent alcohol consumption. *Alcohol Clin Exp Res.* **26**:1114-1119.

27. Madry MM, Kraemer T, Baumgartner MR (2020) Large scale consumption monitoring of benzodiazepines and z-drugs by hair analysis. *J Pharm Biomed Anal.* **183**:113151.

28. Strand MC, Mørland J, Slørdal L, Riedel B, Innerdal C, Aamo T, Mathisrud G, Vindenes V (2017) Conversion factors for assessment of driving impairment after exposure to multiple benzodiazepines/z-hypnotics or opioids. *Forensic Sci Int.* **281**:29-36.

29. Busardò FP, Jones AW (2019) Interpreting γ-hydroxybutyrate concentrations for clinical and forensic purposes. *Clin Toxicol.* **57**:149-163.

30. Lecompte Y, Perrin M, Salle S, Roussel O (2012) Impact of lowering confirmatory test cutoff value in pre-enlistment urine cannabinoids screening: About five years' experience in the French Gendarmerie. *J Anal Toxicol.* **36**:569-574.

31. Druid H, Holmgren P (1997) A compilation of fatal and control concentrations of drugs in postmortem femoral blood. *J Forensic Sci.* **42**:79-87.

32. Fields MD, Abate MA, Hu L, Long DL, Blommel ML, Haikal NA, Kraner J (2015) Parent and metabolite opioid drug concentrations in unintentional deaths involving opioid and benzodiazepine combinations. *J Forensic Sci.* **60**:950-956.

33. Coleman MD (2010) Human drug metabolism: An introduction. Second edition. John Wiley & Sons. West Sussex, UK. Page 8.

34. Caldwell J, Gardner I, Swales N (1995) An introduction to drug disposition: The basic principles of absorption, distribution, metabolism, and excretion. *Toxicol Pathol.* **23**:102-114.

35. Williams JA, Hyland R, Jones BC, Smith DA, Hurst S, Goosen TC, Peterkin V, Koup JR, Ball SE (2004) Drug-drug interactions for UDP-glucuronosyltransferase substrates: A pharmacokinetic explanation for typically observed low exposure (AUC 1/AUC) ratios. *Drug Metab Dispos.* **32**:1201-1208.

36. Garza AZ, Park SB, Kocz R (2020) Drug Elimination. StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing. Available at: https://www.ncbi.nlm.nih.gov/books/NBK547662/ (accessed 6.1.2021).

37. Buajordet I, Ebbesen J, Erikssen J, Brørs O, Hilberg T (2001) Fatal adverse drug events: The paradox of drug treatment. *J Intern Med.* **250**:327-341.

38. Jones AW, Kugelberg FC, Holmgren A, Ahlner J (2011) Drug poisoning deaths in Sweden show a predominance of ethanol in mono-intoxications, adverse drug-alcohol interactions and poly-drug use. *Forensic Sci Int.* **206**:43-51.

39. Kaplowitz N (2004) Drug-induced liver injury. *Clin Infect Dis.* **38**:44-48.

40. Kalgutkar AS, Soglia JR (2005) Minimising the potential for metabolic activation in drug discovery. *Expert Opin Drug Metab Toxicol.* **1**:91-142.

41. Knowles SR, Uetrecht J, Shear NH (2000) Idiosyncratic drug reactions: The reactive metabolite syndromes. *Lancet* **356**:1587-1591.

42. Baillie TA, Cayen MN, Fouda H, Gerson RJ, Green JD, Grossman SJ, Klunk L, LeBlank B, Perkins DG, Shipley LA (2002) Contemporary issues in toxicology: drug metabolites in safety testing. *Toxicol Appl Pharmacol.* **182**:188-196.

43. European Medicines Agency (2013) ICH Topic M3 (R2) Non-Clinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals. European Medicines Agency. London, UK: 2009, updated 2013. CPMP/ICH/286/95. Available at: https://www.ema.europa.eu/en/documents/scientific-guideline/ich-guideline-m3r2-non-clinical-safety-studies-conduct-human-clinical-trials-marketing-authorisation_en.pdf (accessed 6.1.2021).

44. US Food and Drug Administration (2020) Safety testing of drug metabolites: guidance for industry. US Department of Health and Human Services Food and Drug Administration. Center for Drug Evaluation and Research (CDER). Pharmacology/Toxicology. Available at: https://www.fda.gov/media/72279/download (accessed 6.1.2021).

45. Verstraete AG (2004) Detection times of drugs of abuse in blood, urine, and oral fluid. *Ther Drug Monit.* **26**:200-205.

46. Liu RH (1992) Important Considerations in the Interpretation of Forensic Urine Drug Test Results. *Forensic Sci Rev.***4**:51.

47. Moeller KE, Kissack JC, Atayee RS, Lee KC (2017) Clinical interpretation of urine drug tests: What clinicians need to know about urine drug screens. *Mayo Clin Proc.* **92**:774-796.

48. Kronstrand R, Nyström I, Andersson M, Gunnarsson L, Hägg S, Josefsson M, Ahlner J (2008) Urinary detection times and metabolite/parent compound ratios after a single dose of buprenorphine. *J Anal Toxicol.* **32**:586-593.

49. Moyer TP, Charlson JR, Enger RJ, Dale LC, Ebbert JO, Schroeder DR, Hurt RD (2002) Simultaneous analysis of nicotine, nicotine metabolites, and tobacco alkaloids in serum or urine by tandem mass spectrometry, with clinically relevant metabolic profiles. *Clin Chem.* **48**:1460-1471.

50. Jones AW, Helander A (1999) Time course and reproducibility of urinary excretion profiles of ethanol, methanol, and the ratio of serotonin metabolites after intravenous infusion of ethanol. *Alcohol Clin Exp Res.* **23**:1921-1926.

51. Ceder G, Jones AW (2001) Concentration ratios of morphine to codeine in blood of impaired drivers as evidence of heroin use and not medication with codeine. *Clin Chem.* **47**:1980-1984.

52. Druid H, Holmgren P, Carlsson B, Ahlner J (1999) Cytochrome P450 2D6 (CYP2D6) genotyping on postmortem blood as a supplementary tool for interpretation of forensic toxicological results. *Forensic Sci Int.* **99**:25-34.

53. Dettmer K, Aronov PA, Hammock BD (2007) Mass spectrometry-based metabolomics. *Mass Spectrom Rev.* **26**:51-78.

54. Beger RD, Dunn W, Schmidt MA, Gross SS, Kirwan JA, Cascante M, Brennan L, Wishart DS, Oresic M, Hankemeier T, Broadhurst DI, Lane AN, Suhre K, Kastenmuller G, Sumner SJ, Thiele I, Fiehn O, Kaddurah-Daouk R (2016) Metabolomics enables precision medicine: “A White Paper, Community Perspective”. *Metabolomics* **12**(10).

55. Soussan C, Kjellgren A (2016) The users of Novel Psychoactive Substances: Online survey about their characteristics, attitudes and motivations. *Int J Drug Policy* **32**:77-84.

56. Sutherland R, Bruno R, Peacock A, Lenton S, Matthews A, Salom C, Dietze P, Butler K, Burns L, Barratt MJ (2017) Motivations for new psychoactive substance use among regular psychostimulant users in Australia. *Int J Drug Policy.* **43**:23-32.

57. Peacock A, Bruno R, Gisev N, Degenhardt L, Hall W, Sedefov R, White J, Thomas KV, Farrell M, Griffiths P (2019) New psychoactive substances: challenges for drug surveillance, control, and public health responses. *Lancet* **394**:1668-1684.

58. Brandt SD, Sumnall HR, Measham F, Cole J (2010) The confusing case of NRG-1. *BMJ* **341**:62.

59. Stoller A, Dolder PC, Bodmer M, Hammann F, Rentsch KM, Exadaktylos AK, Liechti M, Liakoni E (2017) Mistaking 2C-P for 2C-B: What a difference a letter makes. *J Anal Toxicol.* **41**:77-79.

60. Minutillo A, Palmi I, Mastrobattista L (2019) The health threat of drugs of abuse adulteration by new psychoactive substances. *Clin Ther.* **170**:425-426.

61. Oliver CF, Palamar JJ, Salomone A, Simmons SJ, Philogene-Khalid HL, Stokes-McCloskey N, Rawls SM (2019) Synthetic cathinone adulteration of illegal drugs. *Psychopharmacology.* **236**:869-879.

62. Linsen F, Koning RPJ, van Laar M, Niesink RJM, Koeter MW, Brunt TM (2015) 4-Fluoroamphetamine in the Netherlands: More than a one-night stand. *Addiction* **110**:1138-1143.

63. Pichini S, Pacifici R, Marinelli E, Busardò FP (2017) European Drug Users at Risk from Illicit Fentanyls Mix. *Front Pharmacol.* **8**:785.

64. Helander A, Bäckberg M, Signell P, Beck O (2017) Intoxications involving acrylfentanyl and other novel designer fentanyls – results from the Swedish STRIDA project. *Clin Toxicol.* **55**:589-599.

65. Qin N, Xiang P, Shen B, Zhuo X, Shi Y, Song F (2019) Application of a validated UHPLC-MS/MS method for 28 fentanyl-analogue and novel synthetic opioids in whole blood in authentic forensic cases. *J Chromatogr B Analyt Technol Biomed Life Sci.* **1124**:82-99.

66. Wilde M, Pichini S, Pacifici R, Tagliabracci A, Busardò FP, Auwärter V, Solimini R (2019) Metabolic pathways and potencies of new fentanyl analogs. *Front Pharmacol.* **10**:238.

67. Kraemer M, Boehmer A, Madea B, Maas A (2019) Death cases involving certain new psychoactive substances: A review of the literature. *Forensic Sci Int.* **298**:186-267.

68. Santos-Toscano R, Guirguis A, Davidson C (2020) How preclinical studies have influenced novel psychoactive substance legislation in the UK and Europe. *Br J Clin Pharmacol.* **86**:452-481.

69. Meyer MR (2016) New psychoactive substances: an overview on recent publications on their toxicodynamics and toxicokinetics. *Arch Toxicol.* **90**:2421-2444.

70. Directive (EU) 2017/2103 of the European Parliament and of the Council of 15 November 2017 amending Council Framework Decision 2004/757/JHA in order to include new psychoactive substances in the definition of 'drug' and repealing Council Decision 2005/387/JHA. Available at: https://eur-lex.europa.eu/legal-content/en/TXT/?uri=CELEX:32017L2103#ntr6-L_2017305EN.01001201-E0006 (accessed 6.1.2021).

71. Regulation (EU) 2017/2101 of the European Parliament and of the Council of 15 November 2017 amending Regulation (EC) No 1920/2006 as regards information exchange on, and an early warning system and risk assessment procedure for, new psychoactive substances. Available at: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32017R2101 (accessed 6.1.2020).

72. European Monitoring Centre for Drugs and Drug Addiction (2019), European Drug Report 2019: Trends and Developments, Publications Office of the European Union, Luxembourg. Available at: https://www.emcdda.europa.eu/system/files/publications/11364/20191724_TDAT19001ENN_PDF.pdf (accessed 6.1.2021).

73. Pantano F, Graziano S, Pacifici R, Busardò FP, Pichini S (2019) New psychoactive substances: A matter of time. *Curr Neuropharmacol.* **17**:818-822.

74. JRC Science and Policy Reports (2014) Report on characterization of new psychoactive substances. Publications Office of the European Union. Available at: https://ec.europa.eu/jrc/sites/jrcsh/files/jrc-characterisation-new-psychoactive-substances_en.pdf (accessed 7.3.2020).

75. Carvalho M, Carmo H, Costa VM, Capela JP, Pontes H, Remião F, Carvalho F, Bastos M (2012) Toxicity of amphetamines: An update. *Arch Toxicol.* **86**:1167-1231.

76. World Drug Report 2019 (United Nations publication, Sales No. E.19.XI.8). Booklet 4. Available at: https://wdr.unodc.org/wdr2019/en/stimulants.html (accessed 30.8.2020).

77. Miliano C, Serpelloni G, Rimondo C, Mereu M, Marti M, De Luca MA (2016) Neuropharmacology of New psychoactive substances (NPS): Focus on the rewarding and reinforcing properties of cannabimimetics and amphetamine-like stimulants. *Front Neurosci.* **10**:153. doi: 10.3389/fnins.2016.00153.

78. Barwick V (Ed), Eurachem/CITAC Guide: Guide to quality in analytical chemistry: An aid to accreditation (3rd ed. 2016). ISBN 978-0-948926-32-7. Available at :https://www.eurachem.org/images/stories/Guides/pdf/Eurachem_CITAC_QAC_2016_EN.pdf (accessed 6.1.2021).

79. ISO/IEC Guide 99:2007, International vocabulary of metrology - Basic and general concepts and associated terms (VIM) ISO, Geneva, (2007). Also published (with minor corrections) as JCGM 200:2012. Available at: http://www.bipm.org/en/publications/guides/vim.html (accessed 6.1.2021).

80. Kupiec K, Konieczka P, Namieśnik J (2009) Prospects for the production, research and utilization of reference materials. *Crit Rev Anal Chem.* **39**:311-322.

81. Archer RP, Treble R, Williams K (2011) Reference materials for new psychoactive substances. *Drug Test Anal.* **3**:505-514.

82. Ameline A, Garnier D, Gheddar L, Richeval C, Gaulier JM, Raul JS, Kintz P (2019) Identification and analytical characterization of seven NPS, by combination of (1)H NMR spectroscopy, GC-MS and UPLC-MS/MS®, to resolve a complex toxicological fatal case. *Forensic Sci Int.* **298**:140-148.

83. Wood M, Laloup M, Samyn N, del Mar Ramirez Fernandez, Maria, de Bruijn EA, Maes RAA, Boeck G (2006) Recent applications of liquid chromatography–mass spectrometry in forensic science. *J Chromatogr A.* **1130**:3-15.

84. Sundström M, Pelander A, Ojanperä I (2015) Comparison between drug screening by immunoassay and ultra-high performance liquid chromatography/high-resolution time-of-flight mass spectrometry in post-mortem urine. *Drug Test Anal.* **7**:420-427.

85. Kahl KW, Seither JZ, Reidy LJ (2019) LC-MS-MS vs ELISA: Validation of a comprehensive urine toxicology ccreen by LC-MS-MS and a comparison of 100 forensic specimens. *J Anal Toxicol.* **43**:734-745.

86. Milman BL (2015) General principles of identification by mass spectrometry. *TrAC Trends Anal Chem.* **69**:24-33.

87. Strathmann FG, Lynch KL, Krotulski A, Negri P, Cichelli J, Meyer MR (2020) challenges of high-resolution mass spectrometry for detecting designer drugs. *Clin Chem.* **66**:868-874.

88. Colby JM, Thoren KL, Lynch KL (2018) Suspect screening using LC-QqTOF is a useful Tool for detecting drugs in biological samples. *J Anal Toxicol.* **42**:207-213.

89. Baz-Lomba JA, Reid MJ, Thomas KV (2016) Target and suspect screening of psychoactive substances in sewage-based samples by UHPLC-QTOF. *Anal Chim Acta.* **914**:81-90.

90. Gergov M, Boucher B, Ojanperä I, Vuori E (2001) Toxicological screening of urine for drugs by liquid chromatography/time-of-flight mass spectrometry with automated target library search based on elemental formulas. *Rapid Commun Mass Spectrom*. **15**:521-526.

91. Ibáñez M, Bijlsma L, Van Nuijs ALN, Sancho JV, Haro G, Covaci A, Hernández F (2013) Quadrupole-time-of-flight mass spectrometry screening for synthetic cannabinoids in herbal blends. *J Mass Spectrom.* **48**:685-694.

92. Sundström M, Pelander A, Ojanperä I (2017) Comparison of Post-targeted and Pre-targeted Urine Drug Screening by UHPLC-HR-QTOFMS. *J Anal Toxicol.* **41**:623-630.

93. Noble C, Weihe Dalsgaard P, Stybe Johansen S, Linnet K (2018) Application of a screening method for fentanyl and its analogues using UHPLC-QTOF-MS with data-independent acquisition (DIA) in MSE mode and retrospective analysis of authentic forensic blood samples. *Drug Test Anal.* **10**:651-662.

94. Horai H, Arita M, Kanaya S, Nihei Y, Ikeda T, Suwa K, Ojima Y, Tanaka K, Tanaka S, Aoshima K, Oda Y, Kakazu Y, Kusano M, Tohge T, Matsuda F, Sawada Y, Hirai M, Nakanishi H, Ikeda K, Akimoto N, Maoka T, Takahashi H, Ara T, Sakurai N, Suzuki H, Shibata D, Neumann S, Iida T, Tanaka K, Funatsu K, Matsuura F, Soga T, Taguchi R, Saito K, Nishioka T (2010) MassBank: A public repository for sharing mass spectral data for life sciences. *J Mass Spectrom.* **45**:703-714.

95. mzCloud. Available at: https://mzcloud.org (accessed 5.2.2020).

96. Urbas A, Schoenberger T, Corbett C, Lippa K, Rudolphi F, Robien W (2018) NPS Data Hub: A web-based community driven analytical data repository for new psychoactive substances. *Forensic Chem.* **9**:76-81.

97. Mardal M, Andreasen MF, Mollerup CB, Stockham P, Telving R, Thomaidis NS, Diamanti KS, Linnet K, Dalsgaard PW (2019) HighResNPS.com: An Online Crowd-Sourced HR-MS Database for Suspect and Non-targeted Screening of New Psychoactive Substances. *J Anal Toxicol.* **43**:520-527.

98. Niwa M, Nakai N, Hosaka S, Ishii T, Mayumi T, Ueda A, Yamakawa T, Yasuda Y (2020) Evaluation of the uncertainty of a quantitative approach to drug metabolites in the absence of authentic standards. *Bioanalysis* **12**:7-10.

99. Blanz J, Williams G, Dayer J, Délémonté T, Gertsch W, Ramstein P, Aichholz, Trunzer M, Pearson D (2017) Evaluation of relative MS response factors of drug metabolites for semi-quantitative assessment of chemical liabilities in drug discovery. *J Mass Spectrom.* **52**:210-217.

100. Kruve A (2020) Strategies for drawing quantitative conclusions from nontargeted liquid chromatography-high-resolution mass spectrometry analysis. *Anal Chem.* **92**:4691-4699.

101. Juraschek R, Dülcks T, Karas M (1999) Nanoelectrospray - More than just a minimized-flow electrospray ionization source. *J Am Soc Mass Spectrom.* **10**:300-308.

102. Valaskovic GA, Utley L, Lee MS, Wu J (2006) Ultra-low flow nanospray for the normalization of conventional liquid chromatography/mass spectrometry through equimolar response: Standard-free quantitative estimation of metabolite levels in drug discovery. *Rapid Commun Mass Spectrom.* **20**:1087-1096.

103. Schadt S, Chen L, Bischoff D (2011) Evaluation of relative LC/MS response of metabolites to parent drug in LC/nanospray ionization mass spectrometry: Potential implications in MIST assessment. *J Mass Spectrom.* **46**:1282-1287.

104. ANSI/ASB Standard 036, First Edition (2019), Standard practices for method validation in forensic toxicology. Available at: http://www.asbstandardsboard.org/wp-content/uploads/2019/11/036_Std_e1.pdf (accessed 4.2.2020).

105. Yan X (2006) Unique selective detectors for gas chromatography: Nitrogen and sulfur chemiluminescence detectors. *J Sep Sci.* **29**:1931-1945.

106. Zhang K, Kurita KL, Venkatramani C, Russell D (2019) Seeking universal detectors for analytical characterizations. *J Pharm Biomed Anal.* **162**:192-204.

107. Santos ADC, Dutra LM, Menezes LRA, Santos MFC, Barison A (2018) Forensic NMR spectroscopy: Just a beginning of a promising partnership. *TrAC Trends Anal Chem.* **107**:31-42.

108. Emwas A-M (2015) The strengths and weaknesses of NMR spectroscopy and mass spectrometry with particular focus on metabolomics research. *Methods Mol Biol.* **1277**

109. Kerru N, Gummidi L, Maddila S, Gangu KK, Jonnalagadda SB (2020) A review on recent advances in nitrogen-containing molecules and their biological applications. *Molecules.* **25**:1909.

110. Taylor EW, Qian MG, Dollinger GD (1998) Simultaneous on-line characterization of small organic molecules derived from combinatorial libraries for identity, quantity, and purity by reversed-phase HPLC with chemiluminescent nitrogen, UV, and mass spectrometric detection. *Anal Chem.* **70**:3339-3347.

111. Gudem M, Hazra A (2019) Mechanism of the Chemiluminescent Reaction between Nitric Oxide and Ozone. *J Phys Chem A.* **123**:715-722.

112. Agilent Technologies (2012), Agilent 355 sulfur and 255 nitrogen chemiluminescence detectors. Available at: https://www.agilent.com/cs/library/usermanuals/public/G6600-90006.pdf (accessed 20.12. 2020).

113. Burgett CA, Smith DH, Bente HB (1977) The nitrogen-phosphorus detector and its applications in gas chromatography. *J Chromatogr A.* **134**:57-64.

114. Yan B, Zhao J, Leopold K, Zhang B, Jiang G (2007) Structure-dependent response of a chemiluminescence nitrogen detector for organic compounds with adjacent nitrogen atoms connected by a single bond. *Anal Chem.* **79**:718-726.

115. Winer AM, Peters JW, Smith JP, Pitts JN, Jr. (1974) Response of commercial chemiluminescent NO-NO2 analyzers to other nitrogen-containing compounds. *Environ Sci Technol.* **8**:1118-1121.

116. Felstead DK, Maddison P, Reilly DA (1976) Interferences in the determination of nitrogen dioxide in a chemiluminescent analyser. *Fire Mater.* **1**:123-124.

117. Kashihira N, Tanaka K, Kirita K, Watanabe Y (1980) Gas chromatographic measurement of n-containing compounds. *Bunseki Kagaku.* **29**:35-40.

118. Robbat A, Jr., Corso NP, Liu T (1988) Evaluation of a nitrosyl-specific gas-phase chemiluminescent detector with high-performance liquid chromatography. *Anal Chem.* **60**:173-174.

119. Cai K, Xiang Z, Zhang J, Zhou S, Feng Y, Geng Z (2012) Determination of eight tobacco alkaloids in flue-cured tobacco samples by gas chromatography with nitrogen chemiluminescence detection (NCD). *Anal Methods.* **4**:2095-2100.

120. Fitch WL, Szardenings AK, Fujinari EM (1997) Chemiluminescent nitrogen detection for HPLC: An important new tool in organic analytical chemistry. *Tetrahedron Lett.* **38**:1689-1692.

121. Yan B, Fang L, Irving M, Zhang S, Boldi AM, Woolard F, Johnson CR, Kshirsagar T, Figliozzi GM, Krueger CA, Collins N (2003) Quality control in combinatorial chemistry: Determination of the quantity, purity, and quantitative purity of compounds in combinatorial libraries. *J Comb Chem.* **5**:547-559.

122. Letot E, Koch G, Falchetto R, Bovermann G, Oberer L, Roth H (2005) Quality control in combinatorial chemistry: Determinations of amounts and comparison of the "purity" of LC-MS-purified samples by NMR, LC-UV and CLND. *J Comb Chem.* **7**:364-371.

123. Thomas K, Wechsler D, Chen Y, Crain S, Quilliam MA (2016) Analysis of natural toxins by liquid chromatography-chemiluminescence nitrogen detection and application to the preparation of certified reference materials. *J AOAC Int.* **99**:1173-1184.

124. Rasanen I, Kyber M, Szilvay I, Rintatalo J, Ojanperä I (2014) Straightforward single-calibrant quantification of seized designer drugs by liquid chromatography-chemiluminescence nitrogen detection. *Forensic Sci Int.* **237**:119-125.

125. Rasanen I, Kyber M, Szilvay I, Rintatalo J, Ojanperä I (2019) Single-calibrant quantification of seized synthetic opioids by liquid chromatography-chemiluminescence nitrogen detection. *Forensic Sci Int.* **305**:110001.

126. Ojanperä S, Rasanen I, Sistonen J, Pelander A, Vuori E, Ojanperä I (2007) Quantification of drugs in plasma without primary reference standards by liquid chromatography-chemiluminescence nitrogen detection: application to tramadol metabolite ratios. *Ther Drug Monit.* **29**:423-428.

127. Deng Y, Wu J, Zhang H, Olah TV (2004) Quantitation of drug metabolites in the absence of pure metabolite standards by high-performance liquid chromatography coupled with a chemiluminescence nitrogen detector and mass spectrometer. *Rapid Commun Mass Spectrom.* **18**:1681-1685.

128. Ojanperä S, Tuominen S, Ojanperä I (2007) Single-calibrant quantification of drugs in plasma and whole blood by liquid chromatography-chemiluminescence nitrogen detection. *J Chromatogr B Anal Technol Biomed Life Sci.* **856**:239-244.

129. Kataoka H (1996) Derivatization reactions for the determination of amines by gas chromatography and their applications in environmental analysis. *J Chromatogr A.* **733**:19-34.

130. Flanagan R, Taylor A, Watson I, Whelpton R (2008) Fundamentals of analytical toxicology, Chapter 7: Gas chromatography. 145-176.

131. Meng P, Margot P (2010) Constituent profiling of ecstasy seizures by GC-MS after joint derivatization with MSTFA and MBTFA. *Forensic Toxicol.* **28**:52-54.

132. Meyer MR, Bach M, Welter J, Bovens M, Turcant A, Maurer HH (2013) Ketamine-derived designer drug methoxetamine: metabolism including isoenzyme kinetics and toxicological detectability using GC-MS and LC-(HR-)MSn. *Anal Bioanal Chem.* **405**:6307-6321.

133. Portolés T, Sancho JV, Hernández F, Newton A, Hancock P (2010) Potential of atmospheric pressure chemical ionization source in GC-QTOF MS for pesticide residue analysis. *J Mass Spectrom.* **45**:926-936.

134. Portolés T, Mol JGJ, Sancho JV, Hernández F (2014) Use of electron ionization and atmospheric pressure chemical ionization in gas chromatography coupled to time-of-flight mass spectrometry for screening and identification of organic pollutants in waters. *J Chromatogr A.* **1339**:145-153.

135. Carrasco-Pancorbo A, Nevedomskaya E, Arthen-Engeland T, Zey T, Zurek G, Baessmann C, Deelder AM, Mayboroda OA (2009) Gas chromatography/atmospheric pressure chemical ionization-time of flight mass spectrometry: Analytical validation and applicability to metabolic profiling. *Anal Chem.* **81**:10071-10079.

136. Kind T, Fiehn O (2007) Seven Golden Rules for heuristic filtering of molecular formulas obtained by accurate mass spectrometry. *BMC Bioinform.* **8**:105. https://doi.org/10.1186/1471-2105-8-105.

137. Bade R, Bijlsma L, Miller TH, Barron LP, Sancho JV, Hernández F (2015) Suspect screening of large numbers of emerging contaminants in environmental waters using artificial neural networks for chromatographic retention time prediction and high resolution mass spectrometry data analysis. *Sci Total Environ.* **538**:934-941.

138. Kaufmann A, Butcher P, Maden K, Walker S, Widmer M (2017) Using in silico fragmentation to improve routine residue screening in complex matrices. *J Am Soc Mass Spectrom.* **28**:2705-2715.

139. Tyrkkö E, Pelander A, Ketola RA, Ojanperä I (2013) In silico and in vitro metabolism studies support identification of designer drugs in human urine by liquid chromatography/quadrupole-time-of-flight mass spectrometry. *Anal Bioanal Chem.* **405**:6697-6709.

140. Mollerup CB, Mardal M, Dalsgaard PW, Linnet K, Barron LP (2018) Prediction of collision cross section and retention time for broad scope screening in gradient reversed-phase liquid chromatography-ion mobility-high resolution accurate mass spectrometry. *J Chromatogr A.* **1542**:82-88.

141. Blaženović I, Kind T, Torbašinović H, Obrenović S, Mehta SS, Tsugawa H, Wermuth T, Schauer N, Jahn M, Biedendieck R, Jahn D, Fiehn O (2017) Comprehensive comparison of in silico MS/MS fragmentation tools of the CASMI contest: Database boosting is needed to achieve 93% accuracy. *J Cheminformatics.* **9**:32. https://doi.org/10.1186/s13321-017-0219-x.

142. The National Institute for Standard and Technology, Mass Spectrometry Data Center (2020). Mass spectral library and other tools. Available at: https://chemdata.nist.gov/dokuwiki/doku.php?id=chemdata:start (accessed 21.8.2020).

143. Ruttkies C, Strehmel N, Scheel D, Neumann S (2015) Annotation of metabolites from gas chromatography/atmospheric pressure chemical ionization tandem mass spectrometry data using an in silico generated compound database and MetFrag. *Rapid Commun Mass Spectrom.* **29**:1521-1529.

144. Jordi MA, Rowland K, Liu W, Cao X, Zong J, Ren Y, Liang Z, Zhou X, Louis M, Lerner K (2020) Reducing relative response factor variation using a multidetector approach for extractables and leachables (E&L) analysis to mitigate the need for uncertainty factors. *J Pharm Biomed Anal.* **186**:113334. https://doi.org/10.1016/j.jpba.2020.113334.

145. Orzel J, Krakowska B, Stanimirova I, Daszykowski M (2019) Detecting chemical markers to uncover counterfeit rebated excise duty diesel oil. *Talanta.* **204**:229-237.

146. Deese RD, Morris RE, Metz AE, Myers KM, Johnson K, Loegel TN (2019) Characterization of organic nitrogen compounds and their impact on the stability of marginally stable diesel fuels. *Energy Fuels.* **33**:6659-6669.

147. Wang FC-, Robbins WK, Greaney MA (2004) Speciation of nitrogen-containing compounds in diesel fuel by comprehensive two-dimensional gas chromatography. *J Sep Sci.* **27**:468-472.

148. Toraman HE, Franz K, Ronsse F, Van Geem KM, Marin GB (2016) Quantitative analysis of nitrogen containing compounds in microalgae based bio-oils using comprehensive two-dimensional gas-chromatography coupled to nitrogen chemiluminescence detector and time of flight mass spectrometer. *J Chromatogr A.* **1460**:135-146.

149. McKenzie RM, Özel MZ, Cape JN, Drewer J, Dinsmore KJ, Nemitz E, Hamilton JF, Sutton MA, Gallagher M, Skiba U (2016) The import and export of organic nitrogen species at a Scottish ombrotrophic peatland. *Biogeosciences Discuss.* **13**:2353-2365.

150. Sun S, Li Y, Lv P, Punamiya P, Sarkar D, Dan Y, Ma J, Zheng Y (2016) Determination of prometryn in vetiver grass and water using gas chromatography-nitrogen chemiluminescence detection. *J Chromatogr Sci.* **54**:97-102.

151. Ramírez N, Vallecillos L, Lewis AC, Borrull F, Marcé RM, Hamilton JF (2015) Comparative study of comprehensive gas chromatography-nitrogen chemiluminescence detection and gas chromatography-ion trap-tandem mass spectrometry for determining nicotine and carcinogen organic nitrogen compounds in thirdhand tobacco smoke. *J Chromatogr A.* **1426**:191-200.

152. Moriizumi M, Matsunaga T (2011) Molecular weight separation of hot-water extractable soil organic matter using high-performance size exclusion chromatography with chemiluminescent nitrogen detection. *Soil Sci Plant Nutr.* **57**:185-189.

153. Weijun Y (2015) Direct determination of acrylamide in food by gas chromatography with nitrogen chemiluminescence detection. *J Sep Sci.* **38**:2272-2277.

154. Ozel MZ, Gogus F, Yagci S, Hamilton JF, Lewis AC (2010) Determination of volatile nitrosamines in various meat products using comprehensive gas chromatography-nitrogen chemiluminescence detection. *Food Chem Toxicol.* **48**:3268-3273.

155. Ochiai N, Sasamoto K, MacNamara K (2012) Characterization of sulfur compounds in whisky by full evaporation dynamic headspace and selectable one-dimensional/two-dimensional retention time locked gas chromatography–mass spectrometry with simultaneous element-specific detection. *J Chromatogr A.* **1270**:296-304.

156. Feng D, Liu L, Zhao L, Zhou Q, Tan T (2011) Determination of volatile nitrosamines in latex products by HS-SPME-GC-MS. *Chromatographia.* **74**:817-825.

157. Özel MZ, Hamilton JF, Lewis AC (2011) New sensitive and quantitative analysis method for organic nitrogen compounds in urban aerosol samples. *Environ Sci Technol.* **45**:1497-1505.

158. Jiang Y, Hascall D, Li D, Pease JH (2015) A charged aerosol detector/chemiluminescent nitrogen detector/liquid chromatography/mass spectrometry system for regular and fragment compound analysis in drug discovery. *J Chromatogr A.* **1411**:63-68.

159. Liang X, Patel H, Young J, Shah P, Raglione T (2008) The practical application of implementing the equimolar response principle of chemiluminescent nitrogen detection in pharmaceutical analysis. *J Pharm Biomed Anal.* **47**:723-730.

160. Wachsmuth CJ, Dettmer K, Lang SA, Mycielska ME, Oefner PJ (2014) Continuous water infusion enhances atmospheric pressure chemical ionization of methyl chloroformate derivatives in gas chromatography coupled to time-of-flight mass spectrometry-based metabolomics. *Anal Chem.* **86**:9186-9195.

161. Gornischeff A, Liigand J, Rebane R (2018) A systematic approach toward comparing electrospray ionization efficiencies of derivatized and non-derivatized amino acids and biogenic amines. *J Mass Spectrom.* **53**:997-1004.

162. Mommers J, Ritzen E, Dutriez T, van der Wal S (2016) A procedure for comprehensive two-dimensional gas chromatography retention time locked dual detection. *J Chromatogr A.* **1461**:153-160.

163. Pacchiarotta T, Derks RJ, Hurtado-Fernandez E, Van Bezooijen P, Henneman A, Schiewek R, Fernández-Gutiérrez A, Carrasco-Pancorbo A, Deelder AM, Mayboroda O (2013) Online spectral library for GC-atmospheric pressure chemical ionization-ToF MS. *Bioanalysis.* **5**:1515-1525.